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# Regulation of Heparinase Synthesis in *Flavobacterium heparinum*

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**Abstract.** The effect of various carbon, nitrogen and sulfur sources on the production of heparinase by *Flavobacterium heparinum* in defined medium in the presence and absence of heparin as the inducer has been studied. Carbon catabolite repression has been observed in defined medium containing one of several carbon sources including simple sugars, alcohols and organic acids. Fed batch fermentations result in 10 g/l of cells and heparinase titers as high as 100,000 U/l by avoiding carbon catabolite repression. Growth on heparin as a sole carbon source resulted in both a high growth rate of 0.12 $h^{-1}$ and a high specific activity of 18 U/mg. Specific heparinase activity was markedly reduced when the end products of heparin catabolism were used as carbon, nitrogen or sulfur sources in defined medium. In defined medium with a low sulfate concentration, of less than $10^{-3}$ M, specific activities as high as 8 U/mg have been observed even in the absence of the normally required inducer, heparin.

## Introduction

Heparinase (E.C. 4.2.2.7) is an inducible cell associated enzyme produced by *Flavobacterium heparinum*. Heparinase acts sequentially, along with other flavobacterial enzymes, to catabolize heparin (Dietrich et al. 1973) as shown in Fig. 1. Recent results from this laboratory (Galliher et al. 1981) described a defined growth medium which enhanced the productivity of heparinase 640-fold over that achieved by previous workers (Linker and Hovingh 1972). Interest in the production of heparinase relates to its use for studies involving the elucidation of heparin structure (Linhardt et al. 1982a and Lindahl et al. 1979), blood deheparinization (Langer et al. 1982a), enzymatic assays for heparin (Kanwar and Farquhar 1979) and the preparation of new, heparin derived anticoagulants (Linhardt et al. 1982a).

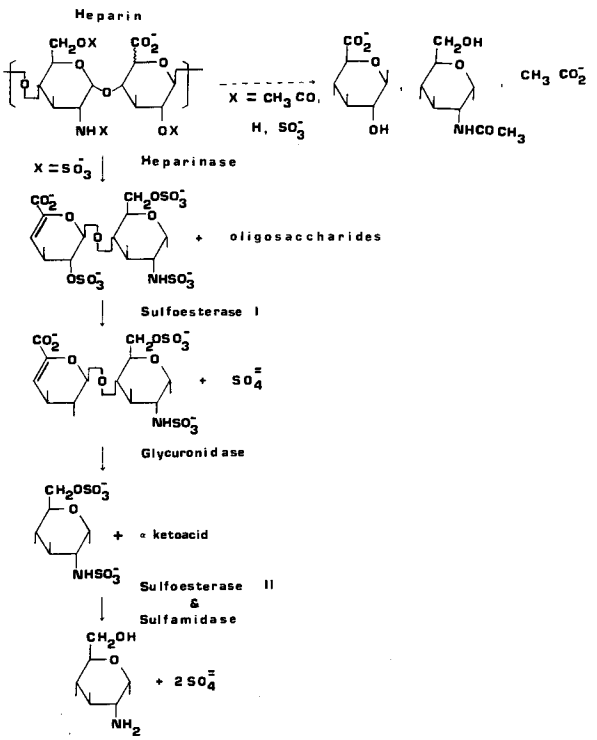


Fig. 1. Proposed sequence of heparin degradation in *F. heparinum* modified from that described by Dietrich et al. 1973. See text for details

The availability of a defined medium for *F. heparinum* facilitates the examination of factors affecting heparinase production. In this paper, results of studies on the effect of carbon, sulfur and nitrogen sources on heparinase production and regulation by the end products of heparin

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catabolism are reported. Most interesting is the observation that in a low sulfate, defined medium, heparinase is produced at a high level in the absence of the inducer heparin. These results provide a new example of an unusually strong sulfate regulation associated with the production of an enzyme. The low sulfate medium obviates the need for added inducer, thus substantially reducing the fermentation costs of producing heparinase.

## Materials and Methods

*Chemicals.* Heparin, as the sodium salt, from porcine intestinal mucosa (Grade II, 153 USP K units/mg), amino acids and 4-nitrocatechol sulfate were from Sigma Chemical Co. Azure A dye was from Fisher Scientific Co. (A-70, certified biological stain, total dye content 70%). All other organic and inorganic chemicals were reagent grade. Media components were obtained from Baltimore Biological Laboratories and Difco Co.

*Analytical Determinations.* Protein was measured by means of the Biuret reaction (Gornal et al. 1949). The protein concentration of the cells was measured by Biuret assay after sonic disruption (Galliher et al. 1981). Reducing sugars were measured with dinitrosalicylic acid (Miller 1959). Assays were performed using a Gilford model 3723 spectrophotometer (Oberlin, Ohio). Heparinase activity and heparin were measured using an azure A dye assay (Galliher et al. 1981). Because of the polydisperse nature of both the substrate and the product it is not possible to relate metachromatic shift to moles of bonds broken; for this reason we have chosen to calculate heparinase activity as mg heparin degraded per hour (see Langer et al. 1982b) for a detailed discussion of assays). Sulfatase activity was determined using a synthetic substrate, 4-nitrocatechol sulfate, as described by (Linhardt et al. 1982b).

*Microorganism. Flavobacterium heparinum,* a soil isolate (Payza and Korn 1956) is a gram-negative, non-motile and non-spore forming rod. Payza and Korn's report of motility is noted in Bergey's Manual (Bergey's Manual, Eighth Edition, 1974), however, no other reports of motility appear in the literature. We have observed no motility with the ATCC 13125 culture, Linker's culture (Linker and Hovingh 1972) and our culture, (Galliher et al. 1981). The microorganism was grown at 23 °C in all experiments. The culture was stored on agar slants as previously described (Galliher et al. 1981)

*Shake Flask Cultivation.* Experiments were conducted in 0.5-l sidearm shake flasks. The defined medium contained (g/l) glucose (sterilized separately), 8.0; $(NH_4)_2SO_4$, 2.0; $K_2HPO_4$, 2.5; $Na_2HPO_4$, 2.5; $MgSO_4 \cdot 7H_2O$ (sterilized separately), 0.5; L-histidine, 0.2 and L-methionine, 0.2 (sterilized separately); heparin, 1.0; trace salts ($Na_2MoO_4 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$) $1 \times 10^{-4}$M and P-2000 antifoam (0.2 ml/l). The shake flasks contained 50 ml of the above medium. L-histidine and L-methionine were added after sterilization with a 0.22 $\mu$m Millipore syringe filter. After sterilization the pH of the flasks was 7.8. Shake flasks were inoculated from slants or from a second shake flask with the culture in exponential growth. A 4 to 6 vol.% inoculum was used and the culture was incubated on a 2.5-cm stroke shaker at 200 rpm. Growth was measured by turbidity in a Klett-Summerson colorimeter (red filter # 66). A Klett unit to dry cell weight (DCW) conversion factor of 260 was obtained. Growth continued until 400–500 Klett units were achieved during which time the pH dropped to 6.0. The cells were harvested or used to inoculate a fermentor with the above medium with a 4 to 6 vol.% inoculum.

*Fermentor Cultivation.* The 2-l fermentor (with a 1.5-l working volume) was equipped with controlled agitation, aeration, pH, temperature, and dissolved oxygen. The fermentor medium was the same as above except for $KH_2PO_4$ (1.0 g/l) and $NaH_2PO_4$ (1.0 g/l). The broth pH was adjusted to 5.0 before steam sterilization for 20 to 25 min at 121° C. After cooling and inoculation, growth, substrate concentration, heparin concentration and heparinase activity were measured. The aeration rate was approximately 0.5 VVM. The pH was controlled at 7.0 ± 0.2 with 1.0 N $NH_4OH$ or 1.0 N $H_2SO_4$ addition. Culture harvest was carried out by centrifugation at 4° C for 10 min at 12,800 g Cell pellets were not washed but were either frozen at −40° C or sonicated within 30 min of centrifugation. Whole cells or crude cell extract from sonication were always kept at 4 °C during handling. All stored cells or extract were kept at -40 °C in polyethylene bottles.

Intermittant fed batch fermentations were carried out as stated above except that additional glucose was added from a presterilized stock solution. Each addition raised the glucose concentration by 10 g/l. This was done twice during the fermentation to obtain approximately 10 g/l dry cell weight.

Continuous culture was performed using the same equipment with the addition of two pumps; the dilution rate was maintained constant by controlling the inlet flow of medium and maintaining constant volume.

## Results and Discussion

### *Carbon Regulation of Heparinase Production*

A defined medium containing heparin (1.0 g/l) and initial glucose concentrations ranging from 3.4 to 68.5 g/l was used to investigate the effect of glucose on growth. Cultures were grown in shake flasks to between 140 and 200 Klett units, harvested and assayed for heparinase activity. A nearly linear decrease in initial growth rate from a maximum of 0.26 $h^{-1}$ with 3.4 g/l of glucose to 0.0 $h^{-1}$ with 68.5 g/l glucose was observed. The growth lag period also increased with increasing glucose concentration. The specific activity of the heparinase obtained was independent of the initial glucose concentration and averaged 13 ± 3.3 U/mg of cell protein (1 U = 1 mg of heparin degraded per hour).

To overcome the adverse effect of high glucose concentration on the growth rate of *F. heparinum* a fed-batch fermentation with defined medium was conducted to maintain glucose concentrations below 15 g/l. The

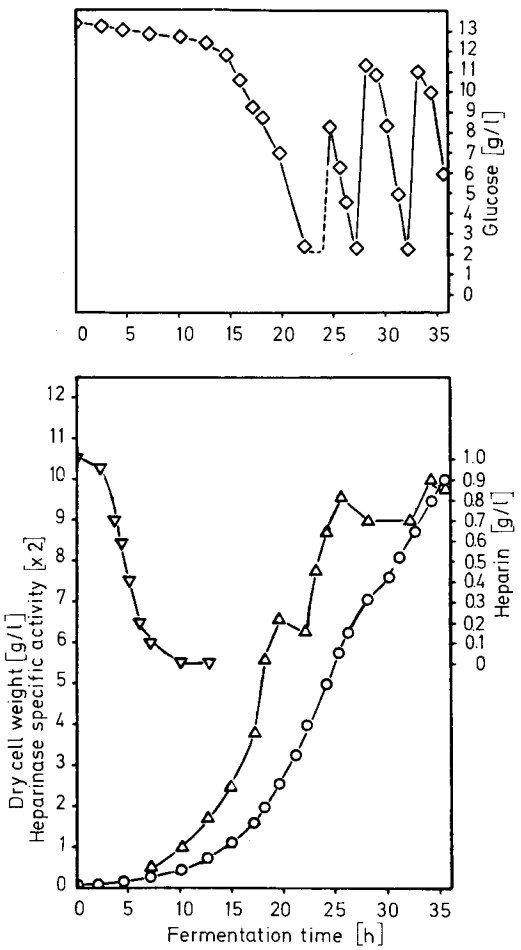


**Fig. 2.** Results of a typical fed-batch fermentation in defined medium showing dry cell weight (○), heparin (▽), glucose (◇), and heparinase specific activity (△) as a function of time as determined in a 2-l fermentor

results, shown in Fig. 2, show a growth rate during heparin degradation of 0.11 $h^{-1}$ followed by a growth rate of 0.18 $h^{-1}$ with glucose alone. A final cell density of 10 g/l was reached with a cell yield of 0.3 g cell/g glucose. After 25 h at 4–6 g/l dry cell weight, the specific growth rate decreased and the specific activity of heparinase reached its maximum, 18 U/mg of cell protein (the protein content was measured as 55% of dry cell weight).

Experiments to examine the effect of carbon sources other than glucose on heparinase production were performed in shake flasks all of which contained defined medium and heparin as the main carbon source at 6.0 g/l. The supplemental carbon source (mono and disaccharide sugars, alcohols or organic acids) was added at 1 and 10 g/l to replace glucose. The flasks were inoculated, from an exponentially growing culture containing 6.0 g/l heparin in defined medium without glucose, grown, harvested and assayed for heparinase activity. Growth on heparin alone gave the highest heparinase specific activity (21 U/mg protein) while addition of chondroitin sulfate A at both 1 and 10 g/l showed decreased activity (13 U/mg protein) although the same growth rate (0.10 $h^{-1}$) was observed. The sugars tested included glucose, maltose, sucrose and N-acetyl-D-glucosamine. At both 1 and 10 g/l all gave reduced heparinase specific activities from 3–11 U/mg protein with approximately the same growth rate (0.10 $h^{-1}$), with the exception of N-acetyl-D-glucosamine at 10 g/l which gave a reduced growth rate of 0.04 $h^{-1}$. The alcohols tested, ethanol and glycerol at both 1 and 10 g/l, showed high heparinase specific activities (8–19 U/mg protein) with a growth rate of 0.10 $h^{-1}$. The addition of acetic or glucuronic acid at 10 g/l gave no growth.

The use of heparin as the sole carbon source results in very high heparinase specific activity. However, the high cost of heparin makes this an expensive way to produce the enzyme. The effect of an additional carbon source on heparinase production during growth on heparin was further studied in a 2-l fermentor. A defined medium initially without glucose and containing 3.0 g/l of heparin was used (Fig. 3). After the initial 3.0 g/l of heparin had been degraded an additional 5.0 g/l heparin was added. At 14 h and at 28 h, glucose was added resulting in a final cell concentration of 5 g/l. The growth rate on heparin was 0.12 $h^{-1}$ while a growth rate of 0.16 $h^{-1}$ was observed on glucose. Heparinase specific activity fell from 18 U/mg to 10 U/mg after glucose addition and then increased to 21 U/mg by the end of the fermentation.

Results in Fig. 2 show that high glucose concentration decreases the growth rate of *F. heparinum* but with minimal adverse effects on heparinase synthesis. These effects can be minimized by fed batch fermentations. At cell densities greater than 6 g/l, the growth rate decreases and the specific activity levels off. Addition of each medium component (including heparin) at this point did not increase growth rate or heparinase specific activity thus indicating that this effect is not the result of a limiting nutrient. One possible explanation of the reduced growth rate is a buildup of inhibitors in the fermentation broth.

The growth of *F. heparinum* on heparin followed by the addition of glucose (Fig. 3) shows that glucose slows heparin degradation and that heparin slows glucose uptake. The addition of glucose also results in a transient decrease in heparinase specific activity from 18 to 10 U/mg followed by a later recovery to 21 U/mg. This effect may be due to a transient catabolite repression by glucose.

The use of alcohols, such as ethanol or glycerol, as supplemental carbon sources resulted in high specific activity with good growth rates indicating that the use of these substrates as sole carbon sources warrants further study. Also, it has been shown that carbon sources which are likely products of heparin catabolism (Fig. 1) such as N-acetyl-D-glucosamine, glucuronic acid and acetic

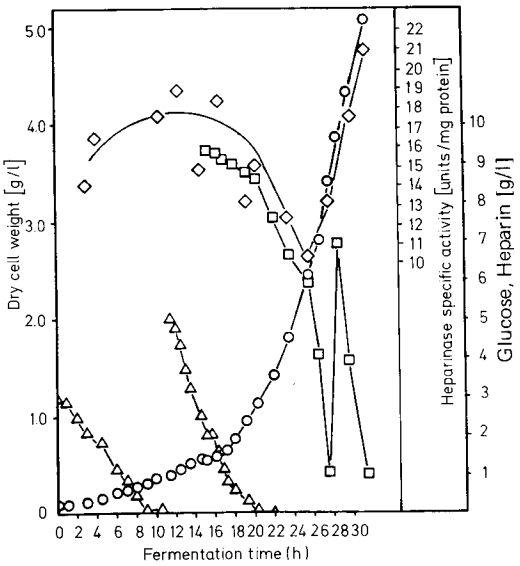


**Fig. 3.** Heparinase specific activity (◇) and dry cell weight (○) in a defined medium fermentation with heparin (△) as the initial carbon source and the effect of late glucose addition (□) in a 2-l fermentor

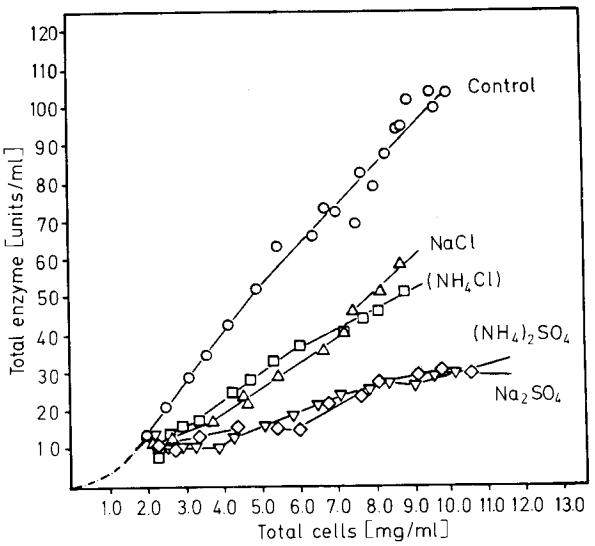


**Fig. 4.** Results of fed-batch fermentations showing total enzyme production as a function of cell concentration in defined medium with no added salts (○); additional $(NH_4)_2SO_4$, 10 g/l (▽); NaCl, 8.8 g/l (△); $NH_4Cl$, 8.1 g/l (□); and $Na_2SO_4$, 10.75 g/l (◇)

acid show the strongest inhibition on the growth rate of *F. heparinum.*

### *Nitrogen Regulation of Heparinase Production*

To examine the nitrogen regulation of heparinase production in *F. heparinum,* shake flasks containing defined medium with heparin over a range of $(NH_4)_2SO_4$ concentrations from 0.5 to 10 g/l (all adjusted to pH 8.0) were studied. The flasks were inoculated with exponentially growing cells from medium without heparin; they were grown for 18 h, harvested and assayed for heparinase activity. This experiment showed a decreasing maximum specific growth rate from 0.21 to 0.12 $h^{-1}$ as well as a decreasing final specific heparinase activity from 6.0 to 0.9 U/mg protein with increasing ammonium sulfate concentration.

A set of experiments was performed in a 2-l fermentor using defined media to differentiate between the ammonium and the sulfate ion effects on heparinase synthesis. The cultures were grown to 2 g/l dry cell weight at which point either $(NH_4)_2SO_4$ (10 g/l, 0.075 M), $NH_4Cl$ (8.1 g/l, 0.15 M), NaCl (8.8 g/l, 0.15 M) or $Na_2SO_4$ (10.75 g/l, 0.075 M) was added after filtering through a sterile 0.22 $\mu$m Millipore syringe filter. Additional glucose was added twice, 10 g/l each time, to obtain a final cell density of about 10 g/l.

Both $Na_2SO_4$ and $(NH_4)_2SO_4$ addition decreased enzyme production to about 3 U/mg-cell while NaCl and $NH_4Cl$ addition decreased enzyme production to 6 U/mg-cell (Fig. 4). The control fermentation without any added salt produced 11.0 U/mg-cell suggesting that high ionic strength may also repress heparinase synthesis. The effect of added $(NH_4)_2SO_4$ appears to be primarily due to the sulfate (a proposed product of heparin catabolism) rather than being a nitrogen effect.

An experiment was performed in shake flasks in which either N-acetyl-D-glucosamine, glucosamine (both proposed products of heparin catabolism) or heparin was used as a nitrogen source instead of $(NH_4)_2SO_4$. When heparin alone was the nitrogen source, a low growth rate (0.03 $h^{-1}$) was obtained, although the specific activity was twice that obtained using $(NH_4)_2SO_4$. These high heparinase titers observed when heparin is used as the sole nitrogen source in place of $(NH_4)_2SO_4$ suggests the presence of a weak nitrogen catabolite repression. However, when N-acetyl-D-glucosamine was used, the growth rates were much higher (0.2 $h^{-1}$) but no heparinase production was observed. With glucosamine, the growth rate was low (0.05 $h^{-1}$) and the heparinase specific activity was one tenth that obtained using $(NH_4)_2SO_4$.

### *End Product Regulation of Heparinase Production*

The most important regulation of heparinase synthesis appears to be its repression by sulfate, a product of heparin catabolism. To examine the role of sulfate in heparinase regulation low sulfate defined medium was prepared

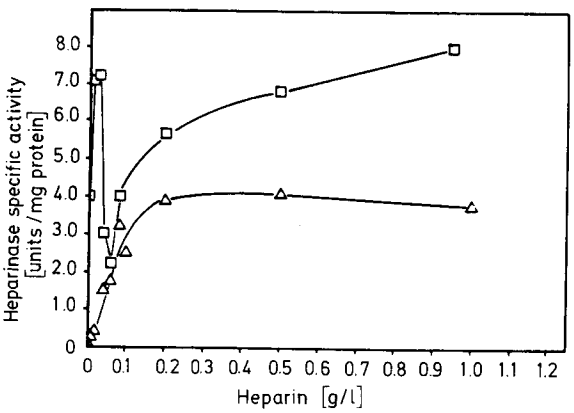


**Fig. 5.** Heparinase specific activity as a function of heparin concentration in low sulfate defined medium (□) and high sulfate defined medium (△). Determined in shake flasks

by replacing $(NH_4)_2SO_4$ with $NH_4Cl$ and $MgSO_4 \cdot 7H_2O$ with $MgCl_2 \cdot 6H_2O$. The standard trace salt mixture was replaced with: $CaCl_2 \cdot 2H_2O$, $FeCl_2 \cdot H_2O$, $CuCl_2 \cdot 2H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$ added to 1.0 liter of preacidified (to pH 2.2 with 0.5 ml of concentrated hydrochloric acid) doubly distilled water. This stock solution was diluted 25 fold upon addition to the shake flask medium to give a final concentration of $10^{-4}$ M for each salt. Doubly distilled water was used in all low sulfate experiments. To study the effect of heparin as a sulfate source, heparin concentration was varied between 0 and 1 g/l in shake flasks using low sulfate medium and the flasks were inoculated with 0.5 ml of exponential culture grown in low sulfate medium without heparin. The cells were grown 10 h into the stationary phase, harvested and assayed for heparinase activity. The results of this experiment are shown in Fig. 5 together with an experiment performed in the standard high sulfate (2 g/l) defined medium. The final cell density was approximately the same in all the flasks (0.86 g/l to 1.05 g/l DCW). High levels of heparinase activity, (4 U/mg), were observed in the low sulfate flask containing no heparin compared to 0.1 U/mg of heparinase in the high sulfate flask containing no heparin. In the low sulfate medium heparinase specific activity increased to 7 U/mg at 0.02 g/l of added heparin then decreased to 2.3 U/mg at 0.06 g/l then increased to 8.0 U/mg at 1 g/l of added heparin. The high sulfate flasks increased from 0.1 U/mg at 0 g/l to 3.9 U/mg at 1.0 g/l added heparin.

The variation of heparin concentration in low sulfate medium (Fig. 5) shows the superimposition of sulfate repression and heparinase induction. An increase in heparin concentration results in an increase in the metabolic products derived from heparin. Among these products is sulfate which may in turn repress heparinase synthesis.

In shake flasks, containing no heparin and at a methionine concentration of 0.2 g/l, the sulfate concentration was varied between $10^{-4}$ and 10 g/l. Heparinase specific activity decreased from 5.5 to 0.01 U/mg-protein as the sulfate concentration was increased from $10^{-4}$ to $10^{-2}$ g/l while the sulfatase specific activity decreased from 5.3 to 1.2 nkatals/mg-protein with the same increase in sulfate concentration. In the presence of heparin (1.0 g/l) at various sulfate concentrations, both heparinase and sulfatase activity show paralled induction profiles. Sulfate appears to repress both heparinase and sulfatase (sulfamidase and sulfoesterase) activity while heparin acts to induce both of these activities, suggesting that these enzymes may be under the same control. A second experiment also performed in shake flasks in low sulfate medium showed that methionine was obligately required in low sulfate medium giving a yield of 58.6 g cells/g methionine. Increased methionine levels did not appreciably affect the synthesis of heparinase. Since heparinase is not repressed by all sulfur sources the sulfate effect on heparinase synthesis is further established as an end product repression.

To study the kinetics of heparinase production without heparin in low sulfate defined medium a fermentor was inoculated with shake flask cultures previously grown and transferred three times in the same low sulfate medium. The concentration of trace sulfate in the fermentor medium was calculated to be $\leqslant 10^{-4}$ g/l. The results given in Fig. 6a show a short lag followed by a growth rate of 0.18 $h^{-1}$ which began to decrease at 1 g/l dry cell weight. Growth continued to a final dry cell weight of 4 g/l. Heparinase activity decreased from an initial 4 U/mg during the rapid initial growth period to 0.23 U/mg after which the specific activity increased to a final level of 7 U/mg. Heparinase synthesis appears to decrease just after inoculation and to increase as the culture slows from exponential to linear growth. These effects may be due to the initial presence and the subsequent depletion of trace quantities of sulfate in the medium.

In a second fermentation (Fig. 6b), a batch culture was grown up in the same fashion for 20 h at which time it was converted to a sulfate-limited continuous culture with a dilution rate of approximately 0.1 $h^{-1}$. This continuous culture was followed for an additional 120 h during which high levels of heparinase specific activity were consistently observed.

These results together with the observation that sulfate does not appreciably inhibit heparinase activity at the concentrations studied ($\leqslant$ 10% at 0.2 M $SO_4^=$ by the methods described in Langer et al. 1981b) suggest strong repression of heparinase synthesis by sulfate. As with other expected products of heparin catabolism by *F. heparinum*, which when acting as a carbon (i.e. glucuronic acid, acetic acid and N-acetyl-D-glucosamine) or nitrogen source (i.e. glucosamine and N-acetyl-D-glucosamine)

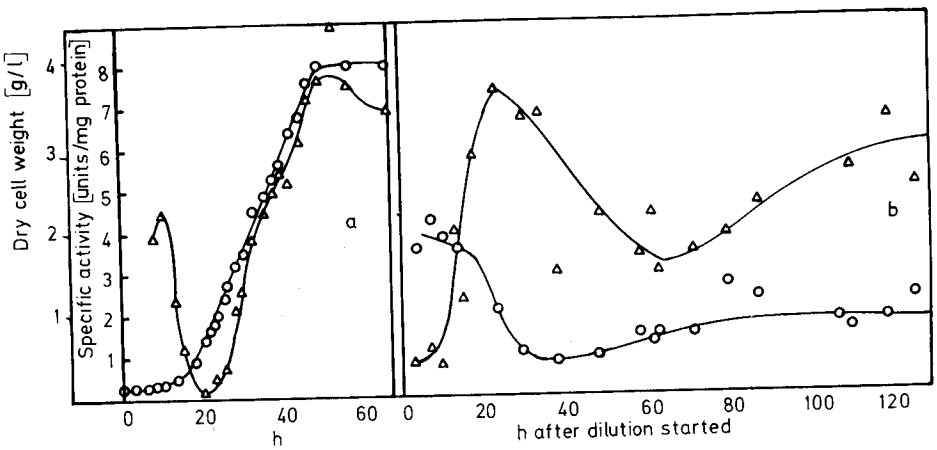


**Fig. 6a.** Results of a fed-batch fermentation in low sulfate defined medium showing cell concentration (○) and heparinase specific activity (△) as a function of time as determined in a 2-l fermentor. **b.** Results of a sulfate limited continuous culture showing cell concentration (○) and heparinase specific activity (△) as a function of time after start of dilution as determined in a 2-l fermentor. The initial dilution rate, 0.1 $h^{-1}$, was changed to 0.09 $h^{-1}$ 37.5 h after the dilution was started

strongly repress heparinase synthesis, sulfate is also a product of this catabolism (Fig. 1) and might also be expected to repress heparinase synthesis.

## Conclusions

Sulfate exerts strong repression of heparinase synthesis. Catabolic products of heparin degradation such as glucosamine, acetate, glucuronic acid, N-acetyl-D-glucosamine and sulfate all appear to repress enzyme synthesis. Significant amounts of heparinase (30–50% of maximum induced levels) can be produced in low sulfate medium without heparin. Some weak repression by glucose and little repression by nitrogen was found. High heparinase titers (100,000 units/l as calculated from the control curve in Fig. 4) and high cell densities (10 g/l) have been obtained using fed batch defined medium with glucose as the carbon source and heparin as the inducer.

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