

Heparinase Production by *Flavobacterium heparinum*

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Heparinase production by *Flavobacterium heparinum* in complex protein digest medium, with heparin employed as the inducer, has been studied and improved. The maximum productivity of heparinase has been increased 156-fold over that achieved by previously published methods to 375 U/liter per h in the complex medium. Rapid deactivation of heparinase activity, both specific and total, was observed at the onset of the stationary phase. Nutritional studies on growth and heparinase production showed an obligate requirement for L-histidine and no vitamin requirement. L-Methionine partially relieved the L-histidine requirement. A defined medium containing glucose, ammonium sulfate, basal salts, L-methionine, and L-histidine was developed for growth and heparinase production. The growth rate in this medium was 0.21 h⁻¹, which is 40% higher than that in complex medium. The maximum volumetric productivity of heparinase in the defined medium was increased to 1,475 U/liter per h, providing a 640-fold increase over that achieved by previously published methods. No rapid deactivation was observed. An examination of alternate inducers for heparinase showed that heparin degradation products, hyaluronic acid, heparin monosulfate, N-acetyl-D-glucosamine, and maltose, induce heparinase in complex medium. An Azure A assay was modified and fully developed to measure the heparin concentration during fermentation and the heparinase specific activity of crude extracts of *F. heparinum* obtained from sonication, thus negating the need for further purification to measure activity."

Heparinase is an inducible, non-extracellular enzyme produced by *Flavobacterium heparinum*. This microorganism was originally selected from soil for its ability to utilize heparin as a sole source of carbon, nitrogen, and sulfur (7). More recent studies were conducted on the production and purification of the flavobacterial enzymes which are responsible for heparin degradation (9). Heparin-induced bacteria contain a series of enzymes, including glycuronidases, sulfoesterases, and sulfamidases as well as heparinase and heparatinase (heparin-monosulfate lyase) (2). Heparinase is an α 1-4-eliminase which acts specifically on the glycosidic linkage between N-sulfated D-glucosamine and sulfated D-glucuronic acid (or L-iduronic acid) present in heparin (11). The elimination reaction at this site, catalyzed by heparinase, results in the formation of smaller polysaccharides with both a reducing end and an α,β -unsaturated acid end group.

Various uses for heparinase (heparin lyase [EC 4.2.2.8]) have been proposed, including the elucidation of the structure of heparin, blood deheparinization (R. Langer, R. J. Linhardt, M. Klein, P. M. Galliher, C. L. Cooney, and M. M. Flanagan, in N. A. Peppas and S. Cooper, ed., *Morphology, Structure and Interaction of*

Biomaterials, ACS Symp. Ser., in press), and enzymatic assays for heparin (6). This enzyme, despite a high demand for it by researchers, has not yet been made commercially available. Several reasons for the unavailability of this enzyme are: failure of the available culture (unpublished data with ATCC 13125) to produce significant amounts of the enzyme and difficulty in obtaining reproducible fermentations; lack of a convenient, sensitive, and reproducible assay to observe heparinase production throughout the fermentation (9); and incomplete knowledge of the factors affecting enzyme production and stability.

In this paper, we report the results of our efforts to establish a simple, reliable method for heparinase production in complex growth medium to investigate compounds, other than heparin, which could induce heparinase, to define the nutritive requirements of *F. heparinum* leading to high levels of heparinase production, and to establish assays suitable for detection of heparinase in both crude cell extracts and purified preparations.

MATERIALS AND METHODS

Chemicals. Heparin, as the sodium salt from porcine intestinal mucosa, was purchased from Sigma Chemical Co., St. Louis, Mo. (grade II, 153 USP K

units per mg). Heparin monosulfate was generously supplied by Thomas Vecchio of The Upjohn Co., Kalamazoo, Mich. All other polysaccharides, amino acids, and vitamins were purchased from Sigma Chemical Co.; Azocoll (50 to 100 mesh) was obtained from Calbiochem, La Jolla, Calif. Azure A dye was purchased from Fisher Scientific Co., Pittsburg, Pa. (A-970; certified biological stain; total dye content, 70%). All inorganic chemicals were reagent grade. Medium components were obtained from BBL Microbiology Systems, Cockeysville, Md., and Difco Laboratories., Detroit, Mich.

Analytical determinations. Protein was measured by the biuret method (3). Reducing sugars were measured with dinitrosalicylic acid (12). Spectrophotometric assays were performed on a Gilford model 3723 spectrophotometer (Gifford Instrument Laboratories, Inc., Oberlin, Ohio).

Azure A assay. A sample of *F. heparinum* to be assayed for heparinase was taken from the fermentation vessel and within 15 min was centrifuged for 10 min at $12,840 \times g$ at 4°C . The supernatant was decanted and the pellet was kept on ice at 4°C if it was to be assayed immediately; the pellet also could be frozen and maintained at -40°C for later assay. The pellet was sonicated within 30 min after centrifugation in a Branson Sonifier (model W350; Branson Sonic Power Co., Stamford, Conn.) at a cell protein concentration of 8 mg/ml in a 5-ml volume. Sonication was carried out with a 125-W, 40% duty, pulsed mode for 12 min in a "cold shoulder" cup maintained below 4°C by an ice bath. The crude cell extract was poured into a borosilicate glass culture tube (12 by 75 mm) and kept at 4°C to be assayed within 1 h after sonication. To another culture tube was added 100 μl of assay mix containing heparin (25 mg/ml in 0.25 M sodium acetate-0.0025 M calcium acetate at pH 7.0). Crude cell extract (200 μl) was added, and the tube was blended in a Vortex mixer and incubated at $30 \pm 1^\circ\text{C}$ in a water bath. At various time intervals, 10- μl samples were withdrawn from the assay tube and added to 10 ml of 0.02-mg/ml Azure A dye solution. The dye showed a metachromatic shift from blue to red in the presence of heparin (5). The change in optical density was measured within 1 h at 620 nm and compared with a standard curve of 0 to 8 μg of heparin per ml in assay broth.

Assay for proteolytic activity in complex medium fermentation. Samples of growing culture were taken at 1-h intervals throughout log growth and 10 h into the stationary phase. To 4 ml of Azocoll (5 mg/ml) (8) in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer containing 0.002 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at pH 8.0, a 1-ml sample of sonically treated material (prepared as above with 7 mg of protein per ml) was added. The reaction mixture was incubated on a shaker at $30 \pm 1^\circ\text{C}$. Samples of 250 μl were taken at 15-min intervals for 3 h and added to 750 μl of 10% (wt/wt) trichloroacetic acid, and the absorbance was read within 2 h at 520 nm. The fermentation broth supernatant was assayed in a similar fashion.

Microorganism. Agar slants of *F. heparinum* were generously supplied by Alfred Linker (Veterans Administration Hospital, Salt Lake City, Utah). This bacterium is a gram-negative rod (approximately 1 by

0.3 μm), nonmotile, and nonsporeforming. The microorganism was grown at 23°C in all experiments, unless otherwise stated.

Culture storage. Agar slants of *F. heparinum* were prepared with Trypticase soy broth (BBL Microbiology Systems) without dextrose, 20 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/liter; trace salts ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 10^{-4} M; and Difco agar, 17 g/liter. This medium was autoclaved for 15 min at 15 lb/in² and placed in slanted Hungate tubes. A pure culture was achieved by serial dilution of shake flask cultures and subsequent plating to isolate single colonies; these colonies were streaked onto individual slants, grown for 2 to 3 days, and stored at 4°C for 3 to 4 weeks. Agar slants and plates of defined medium were prepared as stated above, except a medium containing 10^{-4} M trace salts (as above) and the following was used (grams per liter): glucose, 20; heparin, 1.0; $(\text{NH}_4)_2\text{SO}_4$, 3; K_2HPO_4 , 2.5; Na_2HPO_4 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; L-histidine, 0.5; L-methionine, 0.5; and Difco agar, 17.

Culture growth and heparinase production. Experiments were conducted in shake flasks and 2-liter fermentors in complex medium containing: Trypticase soy broth without dextrose (BBL 11774), 17 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/liter (sterilized separately); trace salts (as above at 10^{-4} M); heparin, 1.0 g/liter; and P-2000 antifoam, 2 drops per liter (Dow Chemical Co., Midland, Mich.). Defined liquid medium consisted of 10^{-4} M trace salts (as above) and the following (grams per liter): glucose (sterilized separately), 8.0; $(\text{NH}_4)_2\text{SO}_4$, 2.0; K_2HPO_4 , 2.5; Na_2HPO_4 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (sterilized separately), 0.5; L-histidine, 0.2; L-methionine, 0.2; and heparin, 1.0. The concentration of amino acids and heparin were varied as stated in the text. To produce heparinase, 500-ml side-arm shake flasks containing 50 ml of one of the above media (complex or defined) were prepared without $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and without glucose, L-histidine, or L-methionine (in the case of the defined medium). The flasks were sterilized with 15 lb of steam per in² for 15 min along with stock solutions of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (30 g/liter) and glucose (200 g/liter). The flasks and stock solutions were cooled to 23°C , and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and glucose were added. In the case of the defined medium, L-histidine and L-methionine were always added with presterilized 0.22- μm membrane syringe filters (Millipore Corp., Bedford, Mass.). After sterilization, the pH's of complex and defined medium flasks were 6.8 and 7.8, respectively. Shake flasks were inoculated from slants with a dose of 4 to 6% and incubated at 23°C on a 2.5-cm-stroke shaker at 200 rpm. Growth was measured by turbidity in a Klett-Summerson colorimeter (red filter no. 66). A conversion factor of 325 to 350 Klett units per g of dry cell weight was obtained. Protein represents 60% of the dry cell weight. Growth continued until 400 to 500 Klett units were achieved, during which time the complex medium broth pH increased to 8.0, and the defined medium broth dropped to pH 6.0. The cells were then harvested or used to inoculate (at a dose of 4 to 6%) a fermentor of the above medium, except with 1.0 g of KH_2PO_4 per liter and 1.0 g of NaH_2PO_4 per liter. Only rapidly growing cultures were used as inocula. Fermentors

were equipped with controlled or monitored agitation, aeration, pH, temperature, and dissolved oxygen. In all cases, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and glucose were steam sterilized separately. Heparin was steam sterilized with the rest of the medium. The fermentor pH was adjusted to 5.0 before sterilization. Steam sterilization lasted 20 to 25 min. After cooling and inoculation, growth, substrate concentration, heparin concentration, and heparinase activity were measured. The aeration rate was approximately 0.5 vol/vol per min. The pH was controlled at 7.0 ± 0.2 with the addition of 1.0 N NH_4OH or 1.0 N H_2SO_4 . Culture harvest was carried out by centrifugation at 4°C for 10 min at $12,840 \times g$. Cell pellets were washed twice with 40 mM pH 7.0 phosphate buffer and frozen at -40°C or sonicated within 30 min of centrifugation in polyethylene bottles. Whole cells or crude cell extracts from sonication were always kept at 4°C during handling. All stored cells or extracts were kept at -40°C in polyethylene bottles.

Nutrient requirements of *F. heparinum*. Experiments to elucidate nutritive requirements of *F. heparinum* were carried out in 500-ml side-arm baffled shake flasks containing 50 ml of medium with various amino acids, 0.2 g/liter; glucose, 10 g/liter; $(\text{NH}_4)_2\text{SO}_4$, 3 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/liter; K_2HPO_4 , 2.5 g/liter; Na_2HPO_4 , 2.5 g/liter; trace salts (as above and at 10^{-4} M); and P-2000 antifoam, 2 drops per liter. Vitamin requirement experiments were conducted in shake flasks, using defined medium, with vitamin-free yeast extract (0.2 g/liter) and the following vitamins (0.02 g/liter): niacinamide, *myo*-inositol, thiamine hydrochloride, *p*-aminobenzoic acid, pyridoxine hydrochloride, *d*-biotin, pyridoxal hydrochloride, folic acid, riboflavin, and *d*-pantothenic acid.

Alternate inducers for heparinase. Experiments to evaluate potential heparinase inducers were carried out in 500-ml side-arm shake flasks containing complex medium as previously described. The compounds (Table 1) were added at 1.0 g/liter at the time of inoculation. The cultures were sampled and assayed for heparinase after 20 h. Compounds showing induction were tested again in shake flask experiments with various concentrations of inducer.

RESULTS

Azure A assay. The Azure A standard curve is linear for heparin concentrations of between 0 and 8 $\mu\text{g}/\text{ml}$ per ml and can be represented by the equation: $y = -0.18x + 1.92$, where y is the absorbance (optical density units) and x is the concentration of heparin (micrograms per milliliter). The correlation coefficient (r) was typically 0.998, and the standard deviation (σ) was 0.504.

The specific activity of heparinase (1 U = 1 mg of heparin degraded per h) was determined by measuring the protein concentration with the biuret method (3). The resulting measurements of specific activity gave a standard deviation (σ) which was 8.3% of the mean.

Azure A is a certified biological stain containing a total dye content which varies slightly among batches and with the age of the dye. A

new standard curve was constructed periodically to ensure the accuracy of this assay.

Growth and heparinase production in complex medium. The growth kinetics of a typical *F. heparinum* fermentation in complex medium in a 2-liter fermentor are shown in Fig. 1. Growth was initially exponential, with a maximum specific rate of 0.15 h^{-1} , and a final cell concentration of 1.6 g/liter was reached. The heparin concentration in the broth fell at a maximum specific rate of 1.1 g/g of cell per h. The maximum specific heparinase production rate of the crude cell extract increased to 2.4 U/g of cell per h; the maximum volumetric productivity of heparinase was 375 U/liter per h, based on a 60% protein content of dry cells. Maximum specific heparinase activity, about 8 U/mg of protein, was reached just before the stationary phase, but within 4 h a 90% loss in specific activity and a 86% loss in total activity occurred.

Alternate inducers for heparinase. Compounds tested for heparinase induction (Table 1) were compared with heparin. To better understand heparinase production, shake flask experiments were conducted to show the effect of heparin, heparin monosulfate, hyaluronic acid, and maltose, on induction is shown in Fig. 2. Heparin and enzymatically produced heparin degradation products were equally effective inducers, whereas heparin monosulfate, hyaluronic acid, maltose, and *N*-acetyl-D-glucosamine induced to a lesser degree.

Nutrient requirement of *F. heparinum*. Experiments designed to elucidate amino acid requirements showed that histidine is required for growth. The obligate requirement for L-histidine is shown in Fig. 3. The medium was de-

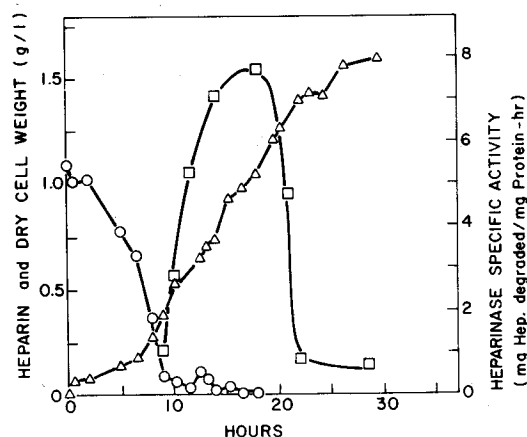


FIG. 1. Results of a typical fermentation on complex medium, showing heparin concentration (\circ), heparinase specific activity (\square), and dry cell weight (\triangle) as functions of time as determined in a 2-liter fermentor.

TABLE 1. *Heparinase inducers*

Compound tested (1 g/liter)	Induction (%)
Heparin, sodium salt	100
Heparin degradation products	100
Heparin monosulfate	23
Chondroitin sulfate C	0
Dextran sulfate	0
Polyvinyl sulfate	0
Hyaluronic acid III	25
Polygalacturonic acid	0
Maltose	3
Sucrose	0
Lactose	0
Cellobiose	0
N-Acetyl-D-glucosamine	3
Glucosamine	0
Glucose	0
Glycerol	0
Galactose	0
Galactosamine	0
D-Glucuronic acid	0
Xylose	0

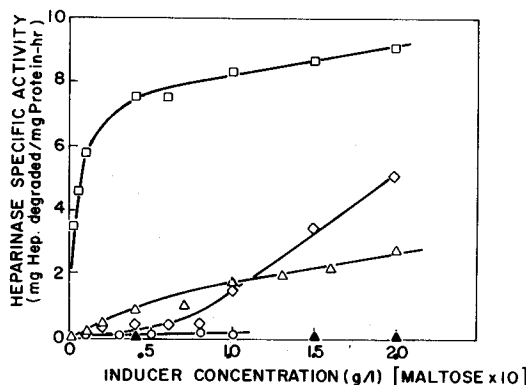


FIG. 2. Specific activity of heparinase in an uninduced control (\blacktriangle) and induced by heparin (\square), hyaluronic acid (\triangle), heparin monosulfate (\diamond), and maltose (\circ) as a function of inducer concentration in shake flasks.

signed so that L-histidine was the growth-limiting factor. A yield of 6.7 g of cell dry weight per g of L-histidine was measured in this experiment. The addition of L-methionine did not relieve the obligate requirement for L-histidine but did increase the growth rate from 0.05 to 0.21 h^{-1} and increased the yield from 6.7 to 50 g of cell dry weight per g of L-histidine. No vitamin requirements were observed.

Defined growth medium and heparinase production. The kinetics of growth and enzyme production by *F. heparinum* on defined medium in a 2-liter fermentor are shown in Fig. 4 and 5. A cell yield on glucose of 0.49 g of cell per g of glucose was measured. The maximum specific heparinase production rate was 0.83 U/g of cell

per h or 1,475 U/liter per h (volumetric). Specific heparinase activity peaked at 16 U. Rapid deactivation of heparinase did not occur in the stationary phase. The heparin concentration decreased at a maximum specific rate of 0.7 g/g of cell per h. The specific glucose uptake rate decreased during heparin degradation and increased after heparin depletion. Growth was ex-

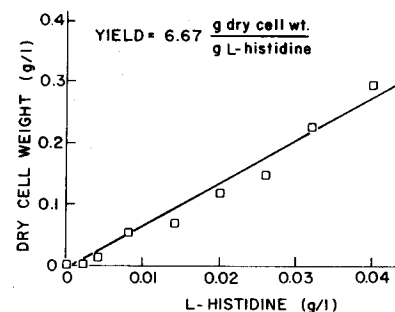


FIG. 3. Final dry cell weight obtained as a function of L-histidine concentrations. Results were determined in duplicate in shake flasks (correlation, $r = 0.986$).

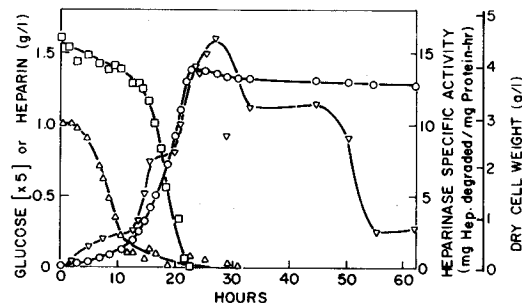


FIG. 4. Results of a typical fermentation on defined medium, showing dry cell weight (\circ), glucose (\square) and heparin (\triangle) concentrations, and heparinase specific activity (∇) as functions of time in a 2-liter fermentor.

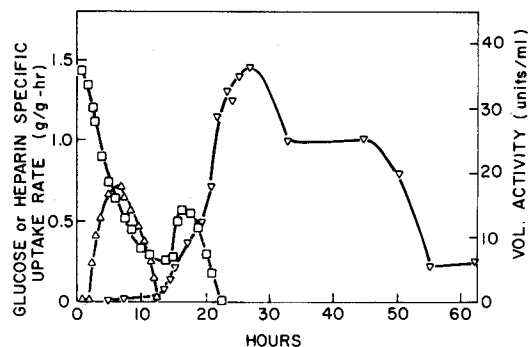


FIG. 5. Specific glucose (\square) and heparin (\triangle) uptake rates and volumetric heparinase activity (∇) as functions of time as determined in a 2-liter fermentor.

potential up to the point of glucose depletion at a maximum specific rate of 0.21 h^{-1} .

DISCUSSION

The use of the Azure A assay, which is simple, fast, and reproducible, was important to the success of this work. Previously published procedures for measuring heparinase activity of crude cell extracts were not satisfactory (7), and due to the existence of contaminating enzymes in the crude preparations, assays used on the purified enzyme proved to be ineffective (9). The Azure A assay has also been useful in measuring the heparin concentration in the fermentation broth because no interference by salts or nutrients was observed.

Jaques (4) has proposed that Azure A dye molecules dimerize in the presence of heparin, resulting in a decrease in π delocalization which is observed as a shift in the absorption maxima, or metachromasia. Assays for heparinase with Azure A can thus be read at either maximum, $\lambda_{\text{max}} = 530$ or $\lambda_{\text{max}} = 620$ nm (5). The latter is used routinely since at this wavelength the slope of the standard curve is greater and the error of the assay is lower. Since heparinase cleaves the α 1-4 linkage in heparin, its action causes chain shortening which results in less Azure A dimerization. The metachromasia of Azure A occurs only in the presence of heparin-derived polysaccharide chains of hexasaccharide size or larger (1). Anticoagulant activity of heparin is also lost by the time chain length is reduced to decasaccharide size (1). Thus, the results of this assay can be used as an estimate of the heparinase activity required to eliminate the biological, anticoagulant activity of heparin.

Volumetric productivity of heparinase of 375 U/liter per h was obtained in complex medium and represents an increase of 156-fold over previously published data (9). This increased production was obtained by controlling environmental parameters and implementing timely induction and harvest procedures. Although Linker and Hovingh (9) listed no value for the activity of their crude heparinase preparation, an estimate of this activity has been made by repeating their purification procedure (9) and correlating the values of specific activity thus obtained (for both the crude and purified heparinases) with the value given by these authors.

An examination of alternate inducers, chosen on the basis of their structural similarity to heparin, with variation in the degree of chain length, sulfation, substitution, and type of polymer linkage, was implemented to provide a better understanding of the importance of structure

of the heparinase inducer. A common feature of inducers is that they contain a glucosamine moiety (except for maltose). Each polysaccharide that induces heparinase contains a 1-4-glycosidic linkage between a glucosamine moiety and hexuronic acid. Those compounds containing a galactose moiety did not induce. The fact that an enzymatic heparin degradation product (10) also induced heparinase suggests that the usual inducer might be a heparin degradation product. The induction capability of hyaluronic acid, containing no sulfation, also suggests that sulfation is not critical to induction.

Fifteen fermentations in complex medium all resulted in high levels of heparinase production, thus demonstrating the reliability and reproducibility of the above method. Though the complex medium is an adequate medium in which to produce high levels of heparinase, there are several problems. A rapid deactivation of heparinase occurs at the onset of the stationary phase, and within 5 h an 86% decrease in total heparinase activity was observed. Since the complex medium is a protein digest, it is likely that single amino acids are utilized for growth first, and once they are depleted, the di-, tri-, and polypeptides are utilized. They most likely require proteases for their utilization, and it was hypothesized that proteases were responsible for heparinase deactivation. Quantitative tests performed throughout the fermentation, however, failed to detect proteolytic activity in either sonicated cell or fermentation broth supernatant samples. As a consequence, an investigation of heparinase deactivation in complex medium is continuing. A second problem in complex medium is that the specific growth rate is lower than that observed in glucose defined medium. This fact and the inactivation of heparinase result in a fourfold reduction in volumetric heparinase productivity. Furthermore, the effects of substrate utilization and nutrient requirements on heparinase production could not be studied easily in complex medium.

The use of defined medium improved the overall volumetric productivity of heparinase by 640-fold over that of previously reported data. In addition, enzyme stability after growth was enhanced; this allowed more flexibility in the harvesting of the product. The specific production rate of heparinase, however, is lower in the presence of glucose, possibly indicating some carbon catabolite repression of heparinase synthesis. The effect of other carbon sources and other factors on growth and heparinase production can be better tested in the defined medium.

In conclusion, this paper describes a reliable method for heparinase production by *F. hepar-*

inum in both complex and defined media. The development of suitable assay methods for heparinase activity and the development of a defined medium provide means for further study of heparinase production in an ongoing effort to increase the availability of this enzyme.

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