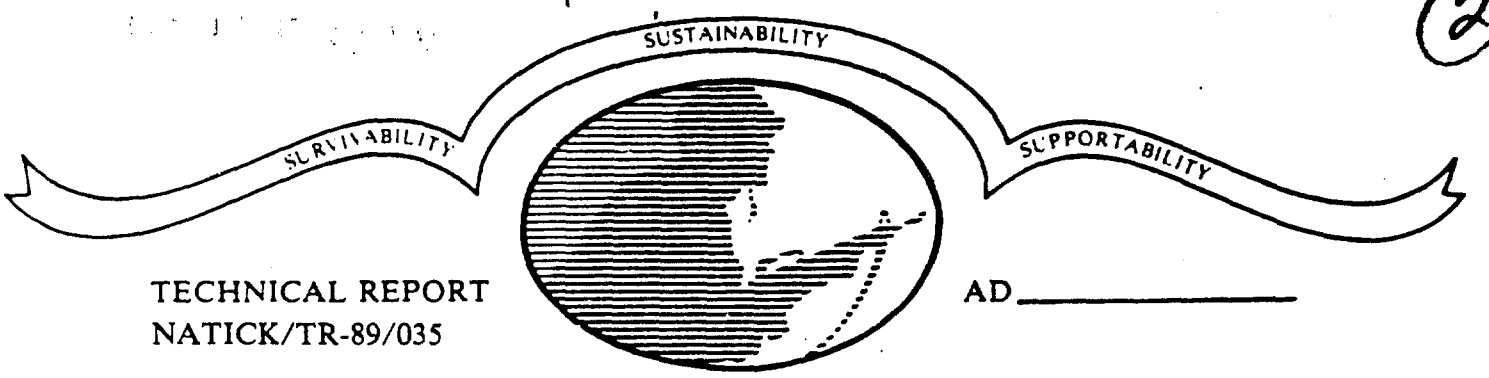

TECHNICAL REPORT
NATICK/TR-89/035

AD_____

PHYSIOLOGICAL REQUIREMENTS FOR THE PRODUCTION OF THE BIOPOLYMER ELSINAN BY SPECIES OF *ELSINOE*

BY

B.J. WILEY
S.M. ARCIDIACONO
D.H. BALL
J.M. MAYER
D.L. KAPLAN

JUNE 1989
FINAL REPORT
JANUARY 1987 - OCTOBER 1988

APPROVED FOR PUBLIC RELEASE;
DISTRIBUTION UNLIMITED

UNITED STATES ARMY NATICK
RESEARCH, DEVELOPMENT AND ENGINEERING CENTER
NATICK, MASSACHUSETTS 01760-5000

SCIENCE AND ADVANCED TECHNOLOGY DIRECTORATE

DISCLAIMERS

The findings contained in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

DESTRUCTION NOTICE

For Classified Documents:

Follow the procedures in DoD 5200.22-M, Industrial Security Manual, Section II-19 or DoD 5200.1-R, Information Security Program Regulation, Chapter IX.

For Unclassified/Limited Distribution Documents:

Destroy by any method that prevents disclosure of contents or reconstruction of the document.

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release: distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NATICK/TR-89/ 035	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION U. S. Army Natick RD&E Center 6b. OFFICE SYMBOL (If applicable) STRNC-YMT	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Natick, MA 01760-5020	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U. S. Army Natick RD&E Center 8b. OFFICE SYMBOL (If applicable) STRNC-YMT	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) Natick, MA 01760-5020	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 61102A; PROJECT NO. 1L161102AH52; TASK NO. 02; WORK UNIT ACCESSION NO. 039

11. TITLE (Include Security Classification)
PHYSIOLOGICAL REQUIREMENTS FOR THE PRODUCTION OF THE BIOPOLYMER ELSINAN BY SPECIES OF ELSINOE

12. PERSONAL AUTHOR(S)
B. J. Wiley, S. M. Arcidiacono, D. H. Ball, J. M. Mayer, and D. L. Kaplan

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM Jan 87 TO Oct 88	1989 June	37

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)			
FIELD	GROUP	SUB-GROUP	BIOPOLYMER;	POLYSACCHARIDE;	CARBON;	MOLECULAR WEIGHT;
			ELSINAN;	FERMENTATION;	NITROGEN;	CHEMICAL PROPERTIES;
			FUNGUS;	ALPHA-LINKED GLUCAN;	PHOSPHATE;	PHYSICAL PROPERTIES;

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

In order to determine optimum environmental conditions for the production of elsinan, the following parameters were evaluated: culture conditions, growth medium, incubation period, pH, and sources and amounts of carbon, nitrogen, and phosphate. A medium was devised for the optimum yields of high (>2 million), medium (1-2 million), and low (<1 million) weight average molecular weight biopolymer products. Batch systems, scale-up batch, and continuous fermentations of one liter and 10 liters were also evaluated, as were processing conditions. Elsinan biopolymer products with weight average molecular weights from less than 500 thousand to over 2 million, with a dispersity of around two were produced. The chemical properties of these fractions were evaluated, but films and fibers were too brittle for physical analyses.

Keywords: culture biology; Biosynthesis;

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	UNCLASSIFIED	
22a. NAME OF RESPONSIBLE INDIVIDUAL Bonnie J. Wiley	22b TELEPHONE (Include Area Code) (508)651-4390	22c. OFFICE SYMBOL STRNC-YNT

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

PREFACE

This report contains the results of a study performed to determine the physiological requirements for the production of the biopolymer elsinan by species of Elsinoe. This study was funded under the U. S. Army Natick Research, Development and Engineering Center (Natick) Program Element 61102A on Biopolymer Products for Varied Military Applications, Project No. 1L161102AH5202039, Task No. 02, Work Unit No. 039. The work was undertaken from January 1987 through September 1988.

We thank Karen Henderson and Robert Stote for their assistance in performing the tangential flow filtration, and Dan Berkowitz, Food Engineering Directorate (FED), for the use of equipment, and his assistance in spray-drying and freeze-drying samples.

Citation of trade names(R) in this report does not constitute an official endorsement or approval of the use of such items.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PHYSIOLOGICAL REQUIREMENTS FOR THE PRODUCTION OF THE BIOPOLYMER ELSINAN BY SPECIES OF *ELSINOE*

INTRODUCTION

Elsinan is a polysaccharide biopolymer released into the growth medium as a secondary metabolite by species of the fungus *Elsinoe*. The biopolymer is a linear α-D-glucan comprised mainly of 1,4- and 1,3-linkages in the molar ratio of ∿2.0 - 2.5:1. One in 140 linkages is α-1,6.[1-4] As described in an earlier study,[5] extracellularly produced biopolymers are the most economical in terms of large-scale production due to the ease of separation, purification, processing, and yield. General properties of many microbial polysaccharides include: an ability to form transparent films with low gas permeability, biodegradability, and excellent strength and flexibility characteristics.[6-9]

The teleomorphic fungus *Elsinoe*, and its anamorphic phase *Sphaceloma* comprise a number of plant pathogenic species, causing spot anthracnoses of citrus, grapes, pear, apple, grasses, tea, dogwood, etc.[10-24] The production of a viscous layer in cultures of *E. australis* was described by Bitancourt and Jenkins in 1936.[12] Other isolates have been described as producing gummy to occasionally mucoid colonies on agar media.[1, 23]

Misaki *et al.*[1-4] produced the polysaccharide that they named elsinan from a culture of *E. leucospila*, and described the characteristics of the biopolymer. Yields of 2.5% - 3.0% of elsinan with weight average molecular weight ranges of 10,000 to 10,000,000 were obtained when the fungus was grown on a medium containing sucrose and potato extract or corn steep liquor. They also examined the effects of various amylolytic enzymes on elsinan and isolated the degradation products.[2, 4] Elsinan forms gels at 5% or higher concentration, and viscosity characteristics of elsinan solutions are affected by temperature. Misaki *et al.*[2] reported that elsinan forms strong films, and has physical properties similar to pullulan. However, few of the metabolic requirements for the production of elsinan have been reported.

In this study, the environmental and nutritional requirements for elsinan production have been studied extensively. The following parameters have been evaluated: culture differences, growth media, pH, sources and amounts of carbon, nitrogen, and phosphate, and incubation period.

The objective of the study was to continue the work on biopolymers: first, to produce and characterize various molecular weight (MW) distribution products of elsinan, and second, to investigate the unique properties of this polysaccharide for potential packaging applications. The characterization of elsinan is the subject of this report.

METHODS AND MATERIALS

Cultures

Eight strains of Elsinoe sp. (Table 1) were evaluated in preliminary studies to compare elsinan production. Strains of Elsinoe fawcettii Jenkins (ATCC 36954 and ATCC 38162), E. tiliae Creelman (ATCC 24510) were chosen for further study, based on product yield and MW, when compared with the other cultures (Table 2). All cultures were maintained on Wickerham's yeast malt extract medium.[25]

Media

The following medium was used, per liter of distilled water: K_2HPO_4, 11.5 mM; NaCl, 34.2 mM; $MgSO_4$, 1.6 mM; $FeSO_4$, 36 μM; Bacto peptone (Difco, Detroit, MI), 0.2% (wt/vol); yeast extract (Difco), 0.2% (wt/vol); carbon source 10% (wt/vol). The carbon source was prepared separately as a 50% solution (wt/vol), and added aseptically after autoclaving.

Culture Conditions

For batch cultures, an Aquaferm Water Bath Shaker(R) (New Brunswick Scientific Co., Inc., Edison, NJ) was set at a temperature of 26°C ± 1°C, and shaken at 125 rpm. An Environ-Shaker(R) (Lab-Line Instruments, Inc., Melrose Park, IL), temperature 26°C ± 1°C was shaken at 125 rpm. A BioFlo Model M30 Fermentor(R) (New Brunswick), temperature 25°C, was set at an agitation rate of 300 rpm, aeration at 0.5 L min^{-1}, or 0.6 L min^{-1}. Media flow-rates were controlled with a Rabbit Peristaltic Pump(R) (Rainin Instrument Co., Woburn, MA) for the continuous fermentation studies. A Model RC-5 Sorvall(R) Refrigerated Centrifuge (du Pont Instruments, Wilmington, DE) with a GSA rotor, 23,400 X g, was used at 10°C, for 20 min, to remove the mycelium.

Preliminary studies were conducted to determine optimum growth conditions for elsinan elaboration. Since the strains of Elsinoe did not sporulate in culture, mycelium was scraped from the surface of agar slants by means of a sterile inoculating loop and transferred to a sterile micro-blender jar (Waring Products Corp., New York, NY). A few milliliters of sterile distilled water were added, and the mycelium was blended for 30 seconds. Aliquots of the resultant slurry of hyphal fragments were pipetted into duplicate 250 mL DeLong(R) flasks containing 50 mL of sterile medium. Cultures were incubated for seven days. The flask contents were centrifuged aseptically, and the supernatant removed. The mycelial pellet was transferred to a sterile micro-blender jar, using approximately 50 mL sterile distilled water to resuspend the mycelium. The mycelium was blended for 15 seconds to form a slurry. All further studies of each Elsinoe strain were conducted using mycelial slurries produced as described.

For dry weight determinations, 1-mL of mycelial slurry was pipetted into each of two 1.5 mL polypropylene centrifuge tubes. The tubes were centrifuged at 25,000 X g for 2 min at ambient temperature in a Microfuge E(R) (Brinkman Instruments, Westbury, NY). The supernatant was decanted, and 1-mL distilled H_2O was added to each tube. Each pellet was resuspended by agitation, using a Vortex Genie(R) (Scientific Instruments, Inc., Bohemia, NJ), and the tubes were centrifuged again. After centrifugation, the supernatant was again decanted, and the tubes were dried at 60°C for 48 hours to determine cell (biomass) dry weights.

Processing and Purification

The culture suspension was transferred aseptically to sterile 250 mL polypropylene centrifuge bottles (Nalge Co., Rochester, NY), then centrifuged at 23,400 X g, 10°C, for 20 min. The supernatant was decanted into a beaker containing ROCCAL II(R) (alkyl dimethyl benzyl ammonium chloride, Sterling Drug, Inc., Montvale, NJ) (1% vol/vol), then the pH was adjusted to pH 7.0 using 5 M NaOH. The elsinan was precipitated from the supernatant, using two volumes of acetone while stirring. After precipitation, the acetone was decanted, the precipitate was washed several times with acetone, and filtered. The elsinan was then air-dried or dried over $CaSO_4$ in a desiccator. Yields of product were determined as percent dry weight of carbon source (wt/wt). The pellet was either retained for future use as inoculum, or dried at 80°C for 72 hours to obtain cell (biomass) dry weights.

All glassware and utensils coming in contact with the live culture were autoclaved to prevent any possible contamination of the environment with this potential plant pathogen.

Larger volumes of culture medium (2 L or more) were purified by using a Tangential Flow Filtration Unit with a Pellicon(R) Model Cassette System OM-141 (Millipore Corp., Bedford, MA). The culture medium was first passed through a 0.45 µm cassette to remove particulate impurities, and then a cassette with a 30,000 MW cut off. The selectively permeable system retained the higher MW biopolymer but allowed the lower MW impurities to pass through. The concentrated retentate was then processed as described above. On one occasion, after Tangential Flow Filtration, the filtrate was freeze-dried overnight, using a Stokes(R) Freeze-Drier (Stokes Vacuum Inc., Philadelphia, PA). Spray drying was also attempted, using a Buchi(R) spray dryer (Brinkman) but the polysaccharide charred onto the glass column as it dried, due to the amount of heat required to dry the sample.

Analytical Methods

Determination of elsinan weight average MW distribution and dispersity was performed as described previously,[5] using a Waters 150-C ALC/GPC Gel Permeation Chromatograph(R) (Waters Chromatography

Div., Millipore Corp., Milford, MA) equipped with a refractive index detector. The system was calibrated using a series of pullulan standards ranging in MW between 12,200 and 853,000 (Polymer Laboratories Ltd., Church Stretton, UK). The standards were run through three Bio-Gel(R) (Bio-Rad Laboratories, Richmond, CA) columns; a TSK-60, separating in the 40,000 to 80,000 MW range, and two TSK-50 columns, effective from 4,000 to 800,000. The TSK-60 column preceded the two TSK-50 columns in line. A third order calibration curve was generated, correlating MW distribution with retention time on the columns. The instrument automatically interpolated the calibration curve and these calculations were used to integrate the area under the sample MW distribution curve to determine weight average MW and dispersity (ratio of weight average molecular weight to number average molecular weight (M_w/M_n). Standards and samples were solubilized at 0.1% (wt/vol) in the carrier solvent, which consisted of either an aqueous solvent of sodium acetate, 0.1 M; acetic acid, 2% (vol/vol), and sodium azide, 0.05% (wt/vol), or a carrier solvent of sodium acetate and sodium azide. The instrument was adjusted to 1.0 mL min^{-1} flow-rate. The injection volume was 200 to 300 µL and the run time was 40 min.

The supernatant was analyzed for free sugars using a High Performance Liquid Chromatograph (HPLC) (Waters) equipped with a Model 6000 solvent delivery system; differential refractometer detector, sensitivity 1 X 10^{-7} r. i. units; Model U6K universal injector, and a Carbohydrate Analysis 30 cm X 3.9 mm I.D. stainless steel column (Waters). Chromatography was performed at ambient temperature (ca. 25°C) using acetonitrile:water mixtures (75:25 or 80:20) with a flow-rate of 2 mL min^{-1}, sample size 5 µL. The system was calibrated using fructose, glucose, and sucrose standards, and standard curves were determined. For quantitation, peak height was measured to determine concentration.

Monomer composition of the isolated polymer was determined after acid hydrolysis using 10 mg of elsinan dissolved in 4 mL of 2 M HCl. The solution was heated under reflux conditions for 3 h. The solution was neutralized by passing it through a small column of Amberlite IR 45 (OH) resin (Rohm and Haas, Philadelphia, PA), then lyophilized and treated with 1 mL Tri-sil Z (Pierce Chemical Co. Rockford, IL). The trimethyl-silyl derivative was analyzed by gas chromatography (Model H-P 5880A Gas Chromatograph, Hewlett-Packard Co., Avondale, PA). The biopolymer identity was confirmed by infrared spectroscopy (5 mg dried elsinan in 200 mg KBr, scan time 24 min, response 1, slit program 7, Model 283 Infrared Spectrophotometer, Perkin-Elmer, Norwalk, CT), and compared with the spectrum given by Misaki, et al.[3]

Film and Fiber Formation

Films were prepared, using 1.6 g of elsinan in 200 mL of 0.1 M NaOH. After the elsinan had solubilized, the pH was adjusted to 5.0. The solution was cast as 15.2 cm X 15.2 cm squares on a plexiglass film-former with a Teflon(R) gasket. After casting, the film was dried at 40°C to 45°C in a forced draft oven. The resultant film was too brittle to perform physical analyses.

RESULTS

Batch Culture

Eight ATCC strains of Elsinoe species (Table 1) were evaluated in preliminary studies in order to determine the best cultures and an optimum growth medium for further evaluation. A modified version of the medium of Misaki et al.[3] was first tried, but the results were not satisfactory. The modified nutrient salts medium used in a previous study[5] yielded varying amounts of product depending on the strain, and this was the medium of choice. Cultures of ATCC 24510, ATCC 36954, and ATCC 38162 had good yields of the biopolymer, with moderate weight average MW products, and these three strains were evaluated further, based on the results shown in Table 2. Since species of Elsinoe are mesophilic in their temperature requirements, a temperature range of $25^{o}C \pm 5^{o}C$ was considered optimum for this fungus.

As shown in Tables 3, 4, and 5, strains of Elsinoe species grow more slowly than most fungi, with optimum yields of the biopolymer produced only after 7 to 10 days of incubation. The time of incubation did not affect the MW of the elsinan product of strains ATCC 36954 or ATCC 24510 (Tables 3 and 5), but did affect the weight average MW of ATCC 38162 (Table 4). Yields of product increased over time with all three strains; ATCC 36954 produced the greatest amounts (Table 3).

As can be seen in Table 3, the strain of ATCC 36954 consistently produced weight average MW fractions of over 2 million, with a dispersity of around 2, and the product yield increased over time. This strain was chosen to assess the environmental and nutritional parameters of pH, and variation in sources and amounts of carbon, nitrogen, and phosphate, as shown in Tables 6 through 12. A pH of 6.0 was chosen for subsequent studies, based on the results shown in Table 6, but other pH levels could have been used as well. Misaki et al.[1-4] have stated that the biopolymer is stable at pH levels from 3.0 to 11.0. After initial adjustment, the pH level was not controlled, and the final pH of the medium was usually around 4.0 in the smaller batch studies. The weight average MW of the product obtained was lower when the higher pH levels were used. All environmental and nutritional parameters, other than carbon source, were studied using a 10% (0.29 M) sucrose concentration.

Figure 1 illustrates the effect of incubation time on elsinan yield and molecular weight distribution of Elsinoe fawcettii ATCC 36954. The highest molecular weight distribution product was attained on the fourth day of incubation, but the highest yield was obtained after 11 days. Biomass dry weights increased exponentially during the first three days of incubation, but increased more slowly over the rest of the period studied. Figure 2 shows the effect of incubation time on residual sugar concentration. Sucrose decreased very slowly until after the fourth day of incubation, when it decreased from 40 mg mL^{-1} to 18 mg mL^{-1} on the fifth day. It then dropped to about 2 mg mL^{-1} on the sixth day, and

then was not detectable during the remainder of the study. Fructose and glucose increased very slowly until the fourth day of incubation, then increased steadily until the ninth day. After that time, the amounts slowly decreased, when the time study was terminated.

The evaluation of various carbon sources is shown in Table 7. Maltose and corn syrup produced elsinan with a MW distribution of about 2 million. However, 0.29 M sucrose was shown in time studies to be a suitable carbon source, and this sugar was chosen, due to economy and availability considerations. Sucrose concentrations of 0.145 M, 0.22 M, and 0.29 M gave comparable percent yields (Tables 8 and 9). Soluble starch as a carbon source gave high yields of product. Yields of product were calculated as percent dry weight of carbon source (wt/wt).

The effects of various sources and amounts of nitrogen on yield and weight average MW biopolymer production are shown in Table 10. Sodium nitrate, or a combination of peptone and yeast extract gave the highest MW distribution of products, and yields were between 15% and 20%, but potassium nitrate was also a good nitrogen source. The use of urea or urea and ammonium sulfate gave a low MW product with yields of from 7% to 10%.

Table 11 shows the effect of varying the phosphate source and concentration. Results were quite variable, with no observable pattern. Yields were best (15% to 16%) with 5.7 mM or 11.5 mM K_2HPO_4, but 7.0 mM and 14 mM Na_2HPO_4 gave yields of about 7%, and moderate weight average MW distribution product. There was an observable effect of potassium phosphate concentration on yield and weight average MW distribution, as shown in Table 12. Concentrations of 5.7 mM through 17.2 mM K_2HPO_4 seemed to have little effect on yield or weight average MW; however, a concentration of 23 mM or 29 mM K_2HPO_4 did affect weight average MW.

Table 13 shows the production of elsinan by 10-liter batch fermentations for varying periods, using a 4% (400 mL) inoculum. The weight average MW was about 1 million. Pigmentation and oxygenation problems occurred with these fermentations, as had occurred with the pullulan 10-liter batch fermentations.[5] The effect of pH may have been a factor, although the red pigment was produced at initial pH values of 6.0 and 7.0, and had not been a problem in the smaller batch studies with these strains.

Continuous Culture

The production of elsinan by continuous fermentation is shown in Table 14. By analyzing the results obtained from the smaller batch studies, an elsinan medium was devised that theoretically should produce optimum yield and MW. The aeration rate used was either 0.5 L min^{-1} or 0.6 L min^{-1}, the agitation rate was 200 or 300 rpm, temperature 26°C ± 1°C, with variable air flow-rates, and varying lengths of incubation. After an initial culture incubation of two to four days, the media flow

was begun. Yields were lower than those produced in the batch studies, and the weight average MW of the product was low, averaging less than 500 thousand.

Extraction, Processing, and Purification

The method previously described for extraction and processing of pullulan[5] was used initially for extracting elsinan. This procedure was modified somewhat when the pigmented product was produced. The culture medium was centrifuged as before to remove cells, then the supernatant was decanted into a beaker without the addition of ROCCAL II. The pH of the solution was adjusted to between 10.0 and 11.0. This mixture was allowed to stand until the pink color of the mixture had faded. The elevated pH helped in the extraction of the pigment and probably changed the pigment structure.[26, 27] Two volumes of acetone were added with stirring, and the precipitate was allowed to settle. The liquid was decanted from the precipitate, and a 60% acetone:40% distilled H_2O solution was added. The pH was adjusted to 7.0, and the product was washed with increasing concentrations of acetone/water to 100% acetone. The product was air-dried, or dried over $CaSO_4$.

Tangential flow filtration was performed on the medium from the large-scale fermentations. One problem that occurred consistently was the presence of a portion of the biopolymer-containing medium that would not pass through the 0.45 μm filter. This fraction was retained for further study.

For further purification of the biopolymer, the dried elsinan was resolubilized in 0.1 M NaOH, the pH adjusted to 7.0, then the solution was centrifuged to remove any extraneous debris. The supernatant was then precipitated as before. Sometimes not all of the biopolymer would go into solution at the high pH, so 5 M HCl was used to lower the pH to below 5.0. Subsequent processing was as described previously.

DISCUSSION

Three strains of Elsinoe (ATCC 36954, ATCC 38162, and ATCC 24510) were selected for further study, based on percent yield and molecular weight distribution of product. Yields of elsinan as high as 25% were produced by Elsinoe fawcettii ATCC 36954 after 10 days of growth, compared with those of E. fawcettii ATCC 38162 (10% to 12%) and E. tilae (10% to 15%); therefore, E. fawcettii ATCC 36954 was used for evaluating environmental and nutritional requirements such as culture conditions, medium constituents, pH, etc.

Production of elsinan by scale-up batch and continuous fermentation resulted in lower yields (up to 9.8%), with weight average molecular weight ranges of from 100 thousand to one million. Pigmentation and oxygenation problems occurred with the large scale fermentations and were

factors that probably affected the yields and weight average molecular weight ranges. These problems still need to be resolved by further study.

This study has shown that with high carbon:nitrogen ratio (150:1 or higher) and appropriate environmental conditions (i.e., pH, variable phosphate concentration, incubation period), controlled molecular weight products can be produced. Sucrose was chosen as the carbon source for evaluating other nutritional parameters, based on availability and economy, but maltose or soluble starch could have been used as well. Peptone, yeast extract, sodium nitrate or potassium nitrate as nitrogen sources gave the highest MW distribution of products. High molar concentrations of K_2HPO_4 (23 mM to 28 mM) gave a high MW distribution of product upon extended incubation (7 to 10 days).

The following environmental conditions were found to be optimal for producing elsinan products in the desired weight average MW:

For high (>2 million) weight average MW product: small batch fermentations (50 mL per 250 mL flask) with short incubation periods of less than five days; elsinan medium with 11.5 mM K_2HPO_4 concentration; unlimited carbon source (10% solutions, wt/vol), the use of Elsinoe fawcettii ATCC 38162 or ATCC 36954, an inoculum of 4% (ca. 10 mg mL^{-1} dry weight), with a pH range from 5.0 to 7.0, and one of several nitrogen sources (i.e., peptone, yeast extract, or sodium nitrate).

For medium (1 - 2 million) weight average MW product: incubation period of from 5 to 7 days; other parameters as above.

For low (<1 million) weight average MW product: large batch fermentations (600 mL per 2800 mL fernbach flask or 10 liter vessels) with incubation periods of over 7 days, or continuous fermentations; elsinan medium; 5 mM urea or 0.2% yeast extract as the nitrogen source; 5.7 mM or 11.5 mM K_2HPO_4 as the phosphate source; the use of soluble starch or sucrose (10% wt/vol) as the carbon source, and other parameters as above.

CONCLUSIONS

This study has shown that by using appropriate environmental conditions, controlled MW elsinan products can be produced. Parameters such as sources and amounts of carbon, nitrogen, and phosphate, pH, and incubation period were manipulated to produce the required MW products. However, films cast from these different MW products were too brittle to allow for the evaluation of physical/chemical properties.

REFERENCES CITED

1. Misaki, A., Y. Tsumuraya, and S. Takaya, A New Fungal α-D-Glucan, Elsinan, Elaborated by Elsinoe leucospila, Agric. Bio. Chem. 42:491-493 (1978).

2. Misaki, A., Y. Tsumuraya, Chapter 11, Structure and Enzymatic Degradation of Elsinan, A New α-D-Glucan Produced by Elsinoe leucospila, p. 197-220, in Fungal Polysaccharides, P. A. Sandford and K. Matsuda (eds.), ACS Symposium Series 126, Am. Chem. Soc., Washington, D. C. 1980.

3. Misaki, A., S. Takaya, K. Yokobayashi, and Y. Tsumuraya, inventors; Director-Gen. of the Tea Experiment Station, Ministry of Agriculture and Forestry, Japanese Government, Shizuoka; A. Misaki, Hyogo, Kabushiki Knisha Hayashibara Seibutsu Kagsku Kenkynja, Okayama, all of Japan, assignees. 1980. Glucan Polysaccharide. U. S. Patent 4,202,966. May 13. 5 p. Int Cl.^{2} C08B 37/00.

4. Misaki, A., H. Nishio, and Y. Tsumuraya, Degradation of Elsinan by alpha-Amylase, and Elucidation of its Fine Structure, Carbohydrate Res. 109:207-219 (1982).

5. Wiley, B. J., S. Arcidiacono, S. Sousa, J. M. Mayer, and D. L. Kaplan, Control of Molecular Weight Distribution of the Biopolymer Pullulan Produced by the Fungus Aureobasidium pullulans, Technical Report, NATICK/TR-88/012, U. S. Army Natick Research, Development and Engineering Center, Natick, MA, 1987.

6. Yuen, S., Pullulan and Its Applications, Process Biochem. 22:7-9 (1974).

7. Yuen, S., Pullulan and Its New Applications (unpublished data, Hayashibara Biochemical Labs., Inc., Japan, 1974).

8. Sugimoto, K., Pullulan: Production and Applications, Fermentation and Industry (Hayashibara Biochemical Labs., Inc., Japan, undated).

9. Yokobayashi, K., and T. Sugimoto, inventors; KK. Hayashibara Seibutsu-Kagaku Kenkyujo, Okayama, Japan, assignee. 1979. Molded Body Consisting of or with a Content of Glucan. German Patent DE28 42 855-A1, 12 April. Int. Cl.^{2}, C08B37/00.

10. Bitancourt, A. A., and A. E. Jenkins, Sweet Orange Fruit Scab Caused by Elsinoe australis, J. Agr. Res. 54:1-18 (1936).

11. Bitancourt, A. A., and A. E. Jenkins, Elsinoe fawcettii, the Perfect Stage of the Citrus Scab Fungus, Phytopath. 26:393-396 (1936).

12. Bitancourt, A. A., and A. E. Jenkins, Perfect Stage of the Sweet Orange Fruit Scab Fungus, Mycologia 28:489-492 (1936).

13. Cunningham, H. S., Histology of the Lesions Produced by Sphaceloma fawcettii Jenkins on Leaves of Citrus, Phytopath. 18:539-545 (1928).

14. Gabel, A. W., and L. H. Tiffany, Host-Parasite Relations and Development of Elsinoe panici, Mycologia 79:737-744 (1987).

15. Jenkins, A. E., Development of the Citrus-Scab Organism Sphaceloma fawcettii, Jour. Agr. Res. 42:545-558 (1931).

16. Jenkins, A. E., Elsinoe on Apple and Pear, Jour. Agr. Res. 44:689-700 (1932).

17. Jenkins, A. E., A Sphaceloma Attacking Navel Orange from Brazil, Phytopath. 23:538-545 (1933).

18. Jenkins, A. E., Additional Studies of Species of Elsinoe and Sphaceloma, Mycologia 25:213-223 (1933).

19. Jenkins, A. E., and A. A. Bitancourt, Studies in the Myriangiales III. Several Little-Know Spot Anthracnoses Affecting Trees, Phytopath. 42:12 (1952).

20. Miller, J. A., Elsinoe on Southern Red Oak, Mycologia 49:277-279 (1957).

21. Tiffany, L. H., and J. H. Mathre, A New Species of Elsinoe on Panicum virgatum, Mycologia 53:600-604 (1961).

22. Todd, E. H., Elsinoe Diseases of Sugarcane in Florida, Plant Disease Rep. 44:153-154 (1960).

23. Zeigler, R. S., and J. C. Lozano, The Relationship of Some Elsinoe and Sphaceloma Species Pathogenic on Cassava and Other Euphorbiaceae in Central and South America, Phytopath. 73:293-300 (1983).

24. Zeller, S. M., and J. W. Deremiah, An Anthracnose of Ledum Caused by a Species of Elsinoe, Phytopath. 21:965-972 (1931).

25. Wickerham, L. J., Taxonomy of Yeasts. Technical Bulletin No. 1029, United States Department of Agriculture, Washington, D. C. 56 p, 5 pl., May 1951.

26. Weiss, U., H. Ziffer, T. J. Batterham, M. Blumer, W. H. L. Hackeng, H. Copier, and C. A. Salemink, Pigments of Elsinoe Species. I. Pigment Production by Elsinoe Species; Isolation of Pure Elsinochromes A, B, and C, Can. J. Microbiol. 11:57-66 (1965).

27. Weiss, U., H. Flon, and W. C. Burger, The Photodynamic Pigment of Some Species of Elsinoe and Sphaceloma, Arch. Biochem. Biophys. 69:311-319 (1957).

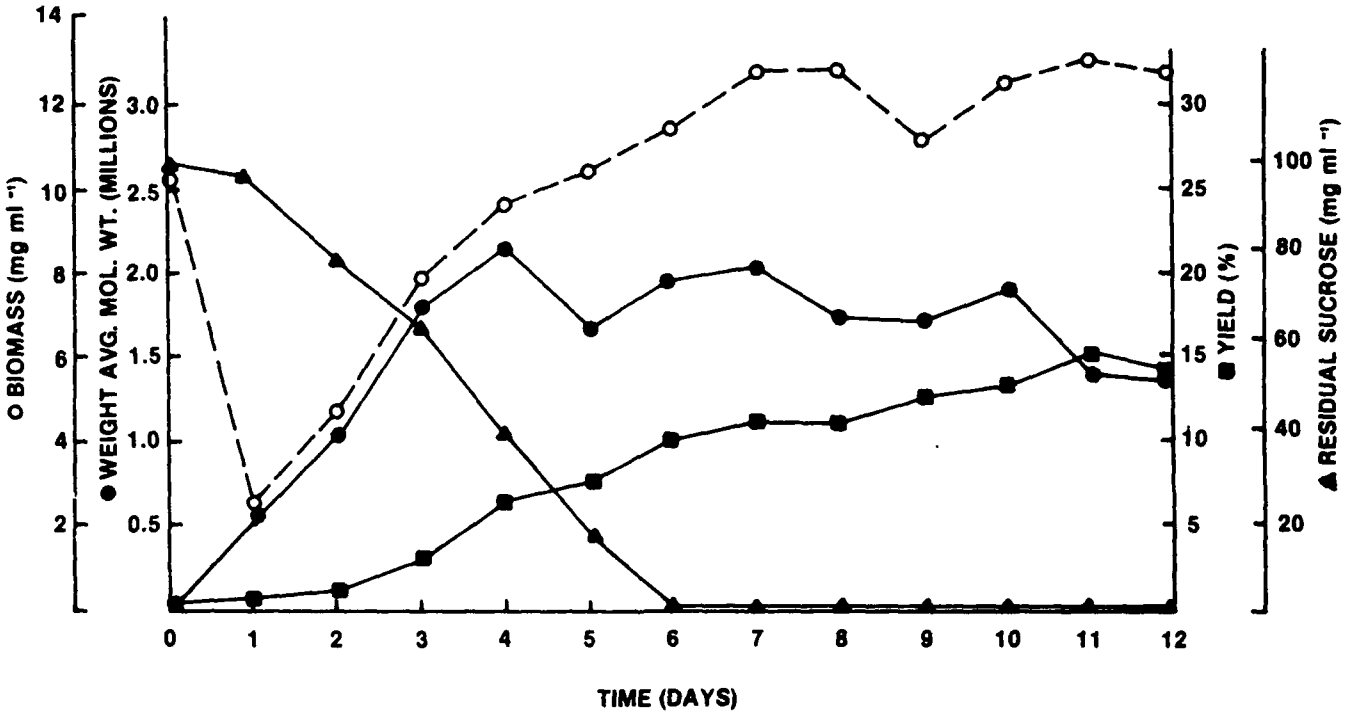

Figure 1. Effect of incubation time on elsinan yield (■) and molecular weight distribution (●) using Elsinoe fawcettii ATCC 36954. Culture conditions: elsinan medium, initial pH 6.0, 11.5 mM K_2HPO_4; 0.29 M sucrose; 26°C ± 1°C; 125 rpm Environ-shaker; 2 flasks, 50 mL per 250 mL Delong flask; five-day-old inoculum (4%) (ca. 10 mg mL^{-1} cell dry wt).

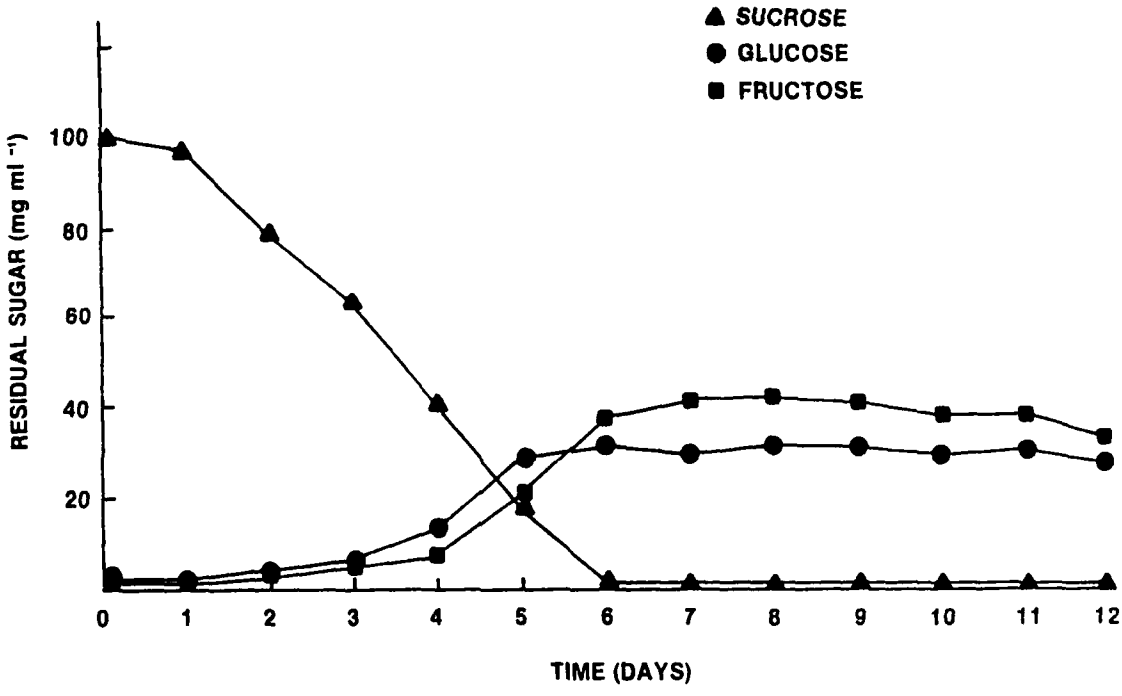

Figure 2. Effect of incubation time on residual sugar concentration. Samples of culture supernatant were analyzed following calibration of the HPLC system using fructose, glucose, and sucrose standards, and determination of standard curves.

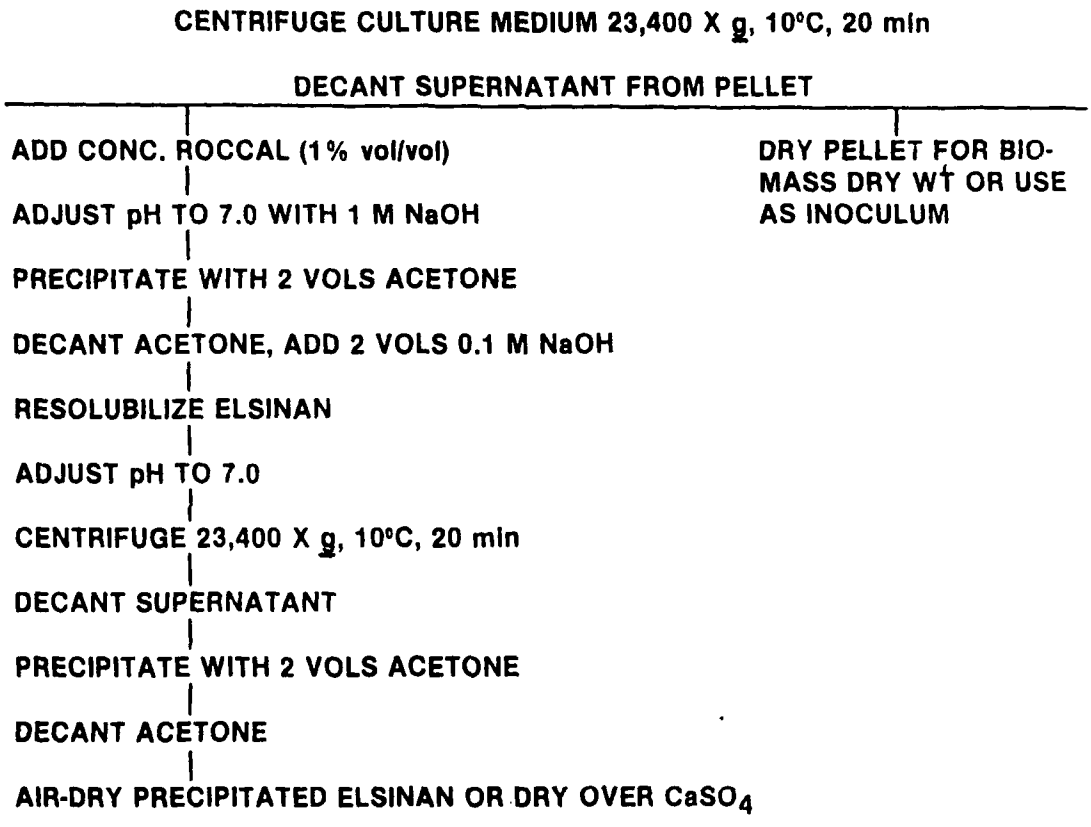

Figure 3. Elsinan processing conditions.

TABLE 1. Fungus Cultures Used for Elsinan Production*

Elsinoe annonae Bitancourt & Jenkins	ATCC 15027
Elsinoe corni Jenkins & Bitancourt	ATCC 11189
Elsinoe fawcettii Jenkins	ATCC 13200
Elsinoe fawcettii Jenkins	ATCC 36954
Elsinoe fawcettii Jenkins	ATCC 38162
Elsinoe heveae Bitancourt & Jenkins	ATCC 12570
Elsinoe lepagei Bitancourt & Jenkins	ATCC 13008
Elsinoe tiliae Creelman	ATCC 24510

*Cultures are available from the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852-1776

TABLE 2. Comparison of ATCC Cultures for Elsinan Elaboration[a]

Sample No.	Culture No.	Incubation (Days)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-49	36954	6	9.6	2052	2.0
E-54	24510	6	15.2	2679	2.4
E-41	11189	6[c]	5.9	2180	1.6
E-59	38162	6	4.5	2347	2.1
E-38	12570	6[c]	2.0	48	2.6
E-40	15027	6	1.8	-	-
E-43	13008	6	0.9	-	-

[a]Culture conditions: Kato & Shiosaka medium, pH 6.0, 0.29 M sucrose; 26°C ± 1°C; 125 rpm shaking water bath or Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong® flask, (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

[c]Red pigment.

TABLE 3. Effect of Incubation Period on Elsinan Yield Using *Elsinoe fawcettii* ATCC 36954[a]

Sample No.	Incubation (Days)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-46	3	6.3	2342	1.4
E-47	4	6.2	2343	1.6
E-48	5	9.3	2351	1.6
E-49	6	9.6	2052	2.0
E-50	7	13.5	2859	2.1
E-62	8	16.6	927	2.1
E-64	9	17.3	1875	1.7
E-66	10	25.4	2330	1.7

[a]Culture conditions: elsinan medium, pH 6.0, 0.29 M sucrose; 26°C ± 1°C; 125 rpm, shaking water bath or Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; 2 mL (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 4. Effect of Incubation Period on Elsinan Yield Using Elsinoe fawcettii ATCC 38162[a]

Sample No.	Incubation (Days)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-56	3	1.5	-	-
E-57	4	1.9	3965	1.5
E-58	5	3.1	3690	1.3
E-59	6	4.5	2347	2.1
E-60	7	4.9	1541	1.7
E-63	8	10.3	2445	1.9
E-65	9	10.3	2572	2.6
E-67	10	12.1	1546	2.0

[a]Culture conditions: elsinan medium, pH 6.0, 0.29 M sucrose; 26°C ± 1°C, 125 rpm, shaking water bath or Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 5. Effect of Incubation Period on Elsinan Yield Using *Elsinoe tiliae* ATCC 24510[a]

Sample No.	Incubation (Days)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-51	3	1.5	2996	4.5
E-52	4	4.1	2149	8.2
E-53	5	10.4	2577	2.6
E-54	6	15.2	2679	2.4
E-55	7	14.8	2369	2.4

[a]Culture conditions: elsinan medium, pH 6.0, 0.29 M sucrose; 26°C ± 1°C, 125 rpm, shaking water bath or Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 6. Effect of pH on Elsinan Yield Using *Elsinoe fawcettii* ATCC 36954[a]

Sample No.	Initial pH	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-73	7.0	18.3	745	1.9
E-74	6.5	14.2	759	2.0
E-75	6.0	14.7	1373	2.2
E-76	5.5	12.4	989	1.8
E-77	5.0	15.2	1449	2.1

[a]Culture conditions: elsinan medium, 0.29 M sucrose; 26°C ± 1°C; 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask, (4%) inoculum, 7 days incubation.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 7. Effect of Carbon Source on Elsinan Yield Using Elsinoe fawcettii ATCC 36954[a]

Sample No.	Carbon Source	(Conc.)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-75	Sucrose	(0.29 M)	14.7	1373	2.2
E-78	Fructose	(0.56 M)	10.9	671	1.8
E-79	Dextrose	(0.56 M)	4.6	1259	1.8
E-80	Lactose	(0.29 M)	14.3	-	-
E-81	Maltose	(0.29 M)	11.8	2250	1.9
E-82	Sol. Starch	(10%)	33.3	774	11.6
E-83	Corn Syrup	(10%)	6.6	2012	1.7

[a]Culture conditions: elsinan medium pH 6.0; 26°C ± 1°C; 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 8. Effect of Sucrose Concentration (A, B, C) on Elsinan Yield Using Elsinoe fawcettii ATCC 36954[a]

Sample No.	Incubation (Days)	Yield g	Yield (%)[b]
A. (0.145 M)			
E-187	3	0.48	9.6
E-189	4	0.60	12.0
E-191	5	0.72	14.4
E-194	6	0.78	15.6
E-196	7	0.77	15.4
E-198	10	0.82	16.4
B. (0.22 M)			
E-188	3	1.07	14.3
E-190	4	0.75	10.0
E-192	5	0.96	12.8
E-195	6	1.10	14.7
E-197	7	1.05	14.0
E-199	10	1.09	14.5
C. (0.29 M)			
E-193	5	1.17	11.7
E-200	10	1.29	12.9

[a]Culture conditions: elsinan medium, pH 6.0; 27°C ± 1°C; 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 9. Effect of Sucrose Concentration (A, B, C) on Elsinan Yield Using Elsinoe fawcettii ATCC 38162a

Sample No.	Incubation (Days)	Yield g	Yield (%)[b]
A. (0.145 M)			
E-172	3	0.26	5.2
E-174	4	0.29	5.8
E-176	5	0.31	6.2
E-179	6	0.42	8.4
E-181	7	0.42	8.4
E-183	10	0.43	8.6
B. (0.22 M)			
E-173	3	0.66	8.8
E-175	4	0.48	6.4
E-177	5	0.56	7.5
E-180	6	0.63	8.4
E-182	7	0.95	12.7
E-184	10	0.75	10.4
C. (0.29 M)			
E-178	5	0.78	7.8
E-185	10	0.99	9.9

[a]Culture conditions: elsinan medium, pH 6.0; 27°C ± 1°C; 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 10. Effect of Nitrogen Source and Concentration on Elsinan Yield Using *Elsinoe fawcettii* ATCC 36954[a]

Sample No.	Nitrogen Source (Conc.)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-86	Yeast Ext. (0.1%)	15.2	155	3.2
E-87	Peptone (0.2%)	4.6	11	1.4
E-88	Urea (5 mM)	10.1	64	2.4
E-89	Urea (5 mM); $(NH_4)_2SO_4$ (1.06 mM)	7.7	496	1.4
E-90	$(NH_4)_2SO_4$ (7.6 mM)	1.8	-	-
E-91	$(NH_4)_2SO_4$ (15.2 mM)	0.8	-	-
E-92	NH_4NO_3 (12.5 mM)	9.4	38	2.2
E-123	Peptone (0.2%); Yeast Ext. (0.02%)	14.6	1803	1.8
E-125	Peptone (0.1%); Yeast Ext. (0.01%)	10.6	1190	5.7
E-126	$NaNO_3$ (23.5 mM)	19.8	755	2.7
E-127	$NaNO_3$ (11.8 mM)	18.2	1581	2.6
E-128	KNO_3 (19.8 mM)	21.1	1564	2.6
E-129	KNO_3 (9.9 mM)	12.9	1674	2.6
E-130	NH_4Cl (37.4 mM)	0.2	-	-
E-131	NH_4Cl (18.7 mM)	0.2	-	-

[a]Culture conditions: elsinan medium, pH 6.0, 0.29 M sucrose; 26°C ± 1°C; 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; 7 days incubation; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 11. Effect of Phosphate Source and Concentration on Elsinan Yield Using Elsinoe fawcettii ATCC 36954[a]

Sample No.	Phosphate Source (Conc.)	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-98	K_2HPO_4 (5.7 mM)	14.8	777	2.2
E-99	K_2HPO_4 (11.5 mM)	16.2	717	2.1
E-103	KH_2PO_4 (11.5 mM)	17.1	1693	2.1
E-139	K_2HPO_4 (5.7 mM)	10.0	113	2.8
E-140	K_2HPO_4 (11.5 mM)	10.8	615	11.0
E-133	Na_2HPO_4 (7.0 mM)	7.9	1363	1.9
E-134	Na_2HPO_4 (14 mM)	7.4	717	4.8
E-135	NaH_2PO_4 (8.3 mM)	8.3	-	-
E-136	NaH_2PO_4 (16.7 mM)	7.1	910	8.8
E-137	Na_2HPO_4 (24.6 mM); NaH_2PO_4 (6.7 mM)	6.4	1077	2.2
E-138	Na_2HPO_4 (12.3 mM); NaH_2PO_4 (3.4 mM)	7.7	134	4.8

[a]Culture conditions: elsinan medium, pH 6.0, 0.29 M sucrose; 26°C ± 2°C; 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; 7 days incubation; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 12. Effect of Phosphate Concentration on Elsinan Yield Using Elsinoe fawcettii ATCC 36954[a]

Sample No.	Phosphate Conc.	Yield (%)[b]	Mol. Wt. (k)	Disp.
	K_2HPO_4 7 Days Incubation			
E-98	5.7 mM	14.8	777	2.2
E-99	11.5 mM	16.2	717	2.1
E-100	17.2 mM	17.7	796	2.1
E-101	23.0 mM	18.0	2135	2.4
E-102	28.7 mM	20.4	1669	2.0
	KH_2PO_4 7 Days Incubation			
E-103	14.7 mM	17.1	1693	2.1
	K_2HPO_4 10 Days Incubation			
E-104	5.7 mM	15.5	848	2.0
E-105	11.5 mM	14.7	829	1.9
E-106	17.2 mM	17.7	708	1.9
E-107	23.0 mM	17.9	594	1.8
E-108	28.7 mM	19.8	1683	1.9
	KH_2PO_4 10 Days Incubation			
E-109	14.7 mM	19.5	620	1.7

[a]Culture conditions: elsinan medium, pH 6.0, 0.29 M sucrose; 26°C ± 4°C, 125 rpm, Environ-Shaker; 2 flasks, 50 mL per 250 mL DeLong flask; (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).

TABLE 13. Production of Elsinan by Scale-up Batch Fermentation Using Elsinoe fawcettii ATCC 36954 and ATCC 38162

Sample No.	Initial pH	Supernatant (mL)	Yield g	Yield (%)[a]	Mol. Wt. (k)	Disp.
E-145	5.5	3000[b]	22.8	7.4	702	77.3
E-142	6.0	3000[c]	5.9	2.0	963	28.5
E-169	6.0	7350[d]	9.7	1.3	978	1.9
E-202	6.5	7730[e]	264.0	34.2	2659	2.9
E-204	7.0	8080[f]	71.3	9.0	1173	2.3

[a]Amount of carbon source converted to elsinan (wt/wt).

[b]Culture conditions: elsinan medium, 600 mL per 2800 mL fernbach flask, 0.29 M sucrose; agitation 125 rpm, 26°C ± 1°C, Environ-Shaker; (4%) inoculum per flask, ATCC 36954, 9 days incubation.

[c]Culture conditions: same as above, 7 days incubation.

[d]Culture conditions: elsinan medium, 10 L Magnaferm fermentor, 0.29 M sucrose; agitation 200 rpm, 27°C, air-flow 5 L min^{-1}; (4%) inoculum, ATCC 38162, 6 days incubation.

[e]Culture conditions: elsinan medium, 10 L Magnaferm fermentor, 0.29 M sucrose; agitation 300 rpm, 2 days; 600 rpm, 4 days; 27°C, air-flow 2.5 L min^{-1}, 1 day, 5 L min^{-1}, 5 days; (4%) inoculum, ATCC 36954, 6 days incubation. Freeze-dried.

[f]Culture conditions: elsinan medium, 10 L Magnaferm fermentor, 0.29 M sucrose; agitation 1200 rpm; 27°C, air-flow 8 L min^{-1}; (4%) inoculum, ATCC 36954, 7 days incubation.

TABLE 14. Production of Elsinan by Continuous Fermentation Using Elsinoe fawcettii ATCC 36954[a]

Sample No.	Initial pH	Flow-Rate (ml hr^{-1})	Supernatant (mL)	Yield g	Yield (%)[b]	Mol. Wt. (k)	Disp.
E-68/	6.0	29	7715	75.5	9.8	451	4.0
E-69						898	3.3
E-70		0	985	2.8	2.2	-	-
E-71		12	2000	12.0	6.0	43	2.0
E-94	6.0	0	1065	4.9	1.7	36	2.1
E-95		12	2265	8.3	3.6	101	2.2
E-118	6.0	25	2700	12.7	4.7	600	9.1
E-119		12	2275	7.9	3.5	-	-
E-120		16	2300	2.1	1.0	92	5.7
E-207	5.5	21	2910	12.2	4.2	197	6.5
E-208		31	6720	49.7	7.4	476	7.0

[a]Culture conditions: elsinan medium, 0.29 M sucrose; 25°C ± 5°C; agitation variable, from 200 to 600 rpm; aeration 0.5 L min^{-1} or 0.6 L min^{-1}, (4%) inoculum.

[b]Amount of carbon source converted to elsinan (wt/wt).