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Large scale production and semi-purification of kedarcidin in a 1000-L fermentor

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SUMMARY

Actinomycete strain ATCC 53650 was grown in a 1000-L fermentor containing 680 L of medium and the production of kedarcidin was monitored by HPLC. The titers of kedarcidin in the fermentor cultures were 0.49–0.53 mg ml^{-1}. A quick and efficient purification method involving the use of anion exchange resin DE23 (batch adsorption–desorption) and an ultrafiltration system yielded high recovery (65% yield) of kedarcidin from the fermentor culture. Over 200 grams of lyophilized kedarcidin of 70% purity was recovered from each of two 1000-L fermentor cultures using this process.

INTRODUCTION

A novel antitumor antibiotic, kedarcidin, was isolated from the supernatant culture medium of an actinomycete strain ATCC 53650 [3,9]. Kedarcidin exhibits potent in vivo antitumor activity against P388 leukemia and B16 melanoma in murine models [9]. Kedarcidin also shows potent activity against Gram-positive bacteria [9]. Structural studies demonstrated that kedarcidin is composed of an acidic polypeptide of 114 amino acid residues and a highly labile enediyne-containing chromophore [3,10]. Kedarcidin belongs to the class of peptide antitumor antibiotics which includes neocarzinostatin [5,11], macromomycin [1,14], C-1027 [4,13] and maduropeptin [2]. It has been demonstrated recently that kedarcidin chromophore cleaves DNA in a sequence-specific manner [17]. Also the kedarcidin apoprotein, which lacks detectable chromophore, possesses selective proteolytic activity [16]. In order to obtain sufficient kedarcidin for expanded biological activity testings and clinical evaluation, large scale fermentation of kedarcidin was carried out at the National Cancer Institute — Frederick Cancer Research and Development Center (FCRDC). This paper describes a large scale fermentation process for production of kedarcidin in the 1000-L fermentors at FCRDC.

MATERIALS AND METHODS

Microorganism

The kedarcidin-producing microorganism was an unidentified actinomycete strain designated ATCC 53650 [9]. Strain ATCC 53650 is related to two genera, *Streptoalloteichus* [15] and *Saccharothrix* [6–8]. Frozen vegetative preparations were maintained in 10% glycerol/5% sucrose solution stored at −80 °C for use as working stocks.

Media

The seed medium used was medium 15734/16 (3% cerelose, 1% Nutrisoy, 1% Pharmamedia and 0.3% $CaCO_3$). The production medium used was medium 48EM (3% glycerol, 1% Pharmamedia, 1.5% Distillers' Solubles extract, 1% fish emulsion (Alaska Fish Fertilizer Co., Renton, WA, USA), 0.05% KH_2PO_4 and 0.6% $CaCO_3$). Distillers' Solubles extract was prepared by heating 10.2 kg Distillers' Solubles in 30 L of water at 90 °C for 2 h. The mixture was filtered and the filtrate was added to the fermentor.

Fermentation conditions

To prepare an inoculum for the production phase, 5 ml of the frozen vegetative stock was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium 15734/16. This seed culture was incubated at 28 °C for 3 days on a rotary shaker set at 250 r.p.m. Fifty milliliters of this seed culture was transferred to a 4-L Erlenmeyer flask containing 1 L of medium 15734/16. This second seed culture was incubated at 28 °C and 250 r.p.m. on a rotary shaker for 3 days. Four liters of the second seed culture was transferred to a 100-L fermentor containing 68 L of medium 15734/16 supplemented with 35 ml of polypropylene glycol P-2000 as antifoam. The third seed culture was incubated at 28 °C, agitation rate of 250 r.p.m. (tip speed: 99 m min^{-1}), aeration of 0.8 v.v.m. and back pressure of 0.35 kg cm^{-2}, for 3 days. Thirty-six liters of the third seed culture was then inoculated into a 1000-L fermentor containing 680 L of medium 48EM

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supplemented with 700 ml polypropylene glycol P-2000 as antifoam. The fermentation was incubated for 6 days under the following conditions: temperature, 28 °C; agitation, 125 r.p.m. (tip speed: 107 m min^{-1}); aeration, 0.7 v.v.m. and back pressure, 0.35 kg cm^{-2}. Polypropylene glycol P-2000 was used as antifoam although no further additions were required during the seed or production fermentation.

Analytical methods

Growth of the organism was determined by the centrifugal solids measured after centrifugation of 10 ml of culture broth at 3000 × *g* for 10 min.

The production of kedarcidin in the fermentation was monitored by HPLC using a TSK-G2000SW column (7.5 × 300 mm, Pharmacia LKB, Piscataway, NJ, USA) and UV absorption at 220 nm. The solvent system was 0.05 M tris-HCl buffer (pH 7.4) with a flow rate of 1 ml min^{-1}. The fermentation broth was centrifuged at 3000 × *g* for 15 min. Three milliliters of the supernatant fluid was passed through an Accell QMA sep-pak cartridge (PN 10835, Waters Associates, Milford, MA, USA) previously equilibrated with 0.05 M sodium acetate buffer (pH 5.6). The cartridge was washed with 10 ml sodium acetate buffer. Kedarcidin was eluted from the cartridge by washing the cartridge with 3 ml of 0.05 M sodium acetate buffer (pH 5.6) containing 0.5 M NaCl. A 50-μl sample of the eluate was used for HPLC analysis. Kedarcidin eluted around 11.6 min.

Anion exchange resins

QMA (Waters), DEAE-Sephadex (Pharmacia LKB), QAE-Sephadex (Pharmacia LKB), DE23 (Whatman, Hillsboro, OR, USA), DE53 (Whatman) and Trisacryl (Pharmacia LKB) were compared for their effectiveness in binding kedarcidin from the culture supernatant fluid of strain ATCC 53650.

Downstream processing for the recovery of kedarcidin from a 1000-L fermentor

Figure 1 summarizes the process for obtaining lyophilized kedarcidin of 70% purity from a 1000-L fermentor. The filter press used was a custom-built horizontal stainless steel plate and frame press manufactured by the Shriver Company (Salt Lake City, UT, USA). The press has 13 plates, each measuring 1.2 × 1.2 m, and has a capacity of approximately 736 L. The press was precoated with a diatomaceous earth (Dicalite, Grefco Co., Torrance, CA, USA) slurry prior to filtration. Dicalite was also used as a body feed add-mix along with the fermentation broth. The basket centrifuge was a stainless steel Bock model 755 (Toledo, OH, USA) with a 76-cm perforate basket. The unit has a capacity of 79 L, and provides centrifugal force of 1100 × *g*.

The hollow fiber ultrafiltration unit (model H53PM10) was a 4.9 m^2 polysulfone self-contained cartridge unit obtained from Amicon Corporation (Beverly, MA, USA). A Wilden (Colton, CA, USA) stainless steel model M-2 air drive double diaphragm pump was used for circulation with a flux rate of 2 L min^{-1}. The lyophilizer used for processing the concentrated kedarcidin solution was a Virtis model 25SRC (Gardiner, NY, USA) tray unit having a total capacity of 10–12 L.

RESULTS AND DISCUSSION

Fermentation of kedarcidin in a 1000-L fermentor

Figure 2 shows the time course of kedarcidin fermentation by strain ATCC 53650 in a 1000-L fermentor containing 680 L of medium 48EM. Results obtained from 50-L laboratory (pilot) fermentations demonstrated that the pH of the fermentor culture rose sharply from 6.9 to 8.4 during the initial 40 h of the fermentation and no kedarcidin was detected in these fermentations. Therefore the pH of the 1000-L production tank fermentation was kept at or below 7.5 by the addition of 10 N H_2SO_4. During the initial 36 h of the fermentation, a substantial amount of acid (4 L) was added to the fermentor to keep the pH of the fermentation at 7.5. The pH of the fermentation then dropped from 7.5 at 36 h to 6.2 at around 54 h. The start of production of kedarcidin was also detected at around 54 h. The production of kedarcidin increased gradually, reaching a maximum titer of 0.53 mg ml^{-1} at 145 h. The centrifugal solids of the fermentation increased from 3.5% at inoculation to 10% at around 42 h. The centrifugal solids of the fermentation remained at about 10% from 42 h to 128 h indicating that strain ATCC 53650 entered the stationary phase at around 42 h. The centrifugal solids of the fermentation started to rise after 128 h. The increase in centrifugal solids at the late stage of the fermentation may be due to thickening of the organism's cell wall and the accumulation of assimilatory materials such as lipids and nonstructural carbohydrates rather than reflecting further growth of the organism [12]. The percentage of CO_2 in the off gas increased from 0 to 0.4% during the initial 22 h of the fermentation, followed by a drop to 0.2% at 32 h and then levelled off at around 0.2% for the rest of the fermentation. Fermentation and production profiles for the second 1000-L fermentor cultivation of strain ATCC 53650 were very similar to those of the first fermentor run. The final titer of kedarcidin in the second fermentor culture was 0.49 mg ml^{-1}.

Evaluation of anion exchange resins for large scale isolation of kedarcidin

Major problems associated with the isolation of a specific extracellular protein from a large-scale fermentation process concern reduction of the large initial volume of liquid to a manageable level, and elimination of the myriad of extraneous proteins from the conditioned medium (i.e. enrichment of the target protein). The first step of this type of isolation usually involves the reduction of the liquid volume containing the protein of interest to a manageable size for later chromatographic purification. Kedarcidin is an acidic chromoprotein with a pI of 3.65. Anion exchange resin in a batch adsoption–desorption mode seems to be a rapid and convenient way of reducing the liquid volume of kedarcidin from the fermentor culture. The choice of anion exchange resin for the above process is very important. Six anion exchange resins were evaluated for their loading capacity and the volume ratio between supernatant fluid and adsorbent.

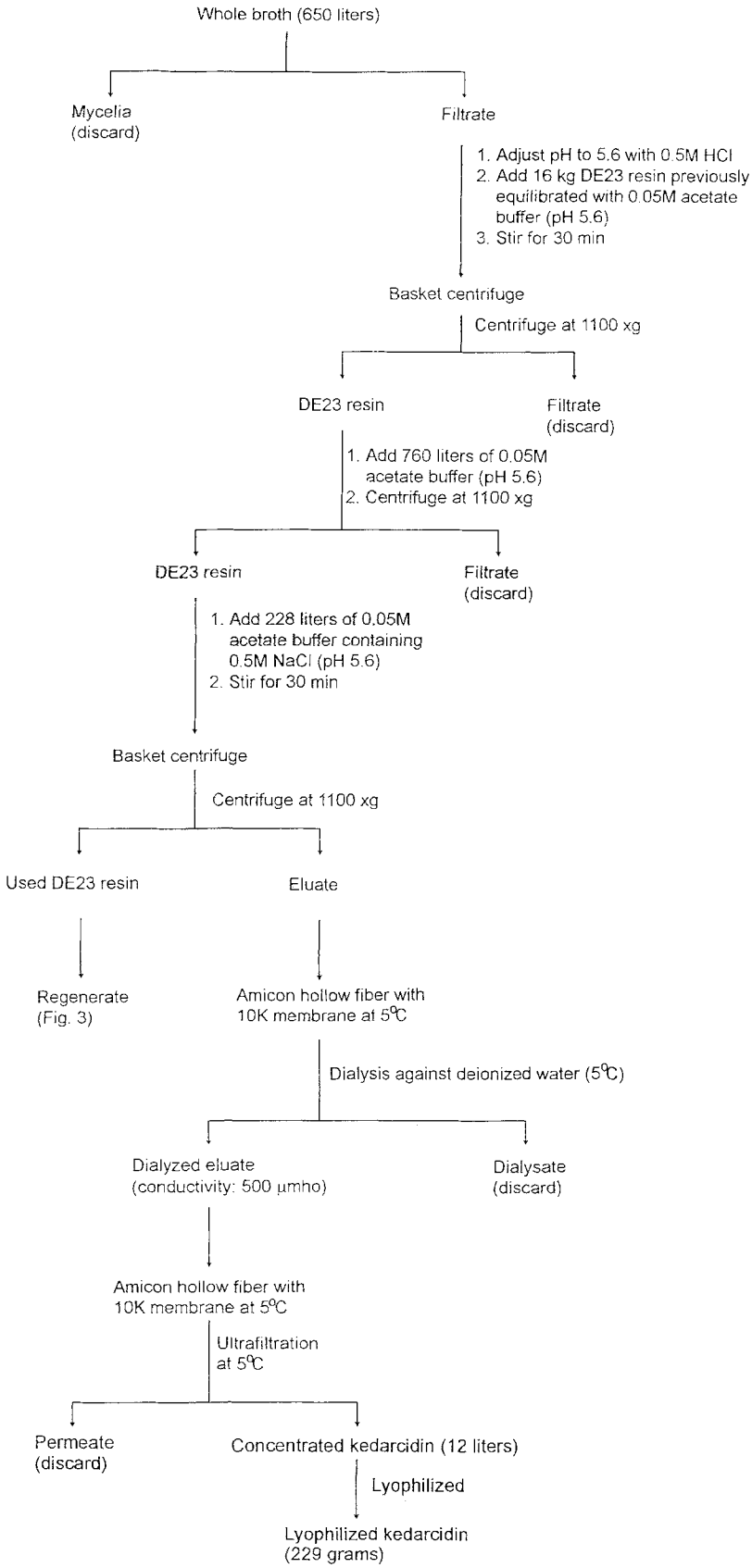

Fig. 1. Downstream processing for the recovery of kedarcidin from a 1000-L fermentor.

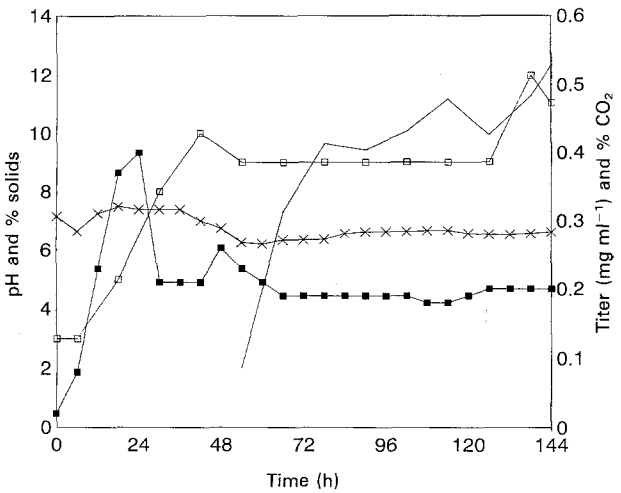

Fig. 2. Time course of kedarcidin fermentation by actinomycete strain ATCC 53650 in a 1000-L fermentor. Symbols: -■-, % CO_2; -□-, centrifugal solids; -*-, pH and -+-, titer of kedarcidin.

One milliliter of the supernatant medium from the kedarcidin fermentation was sequentially and cumulatively added to each adsorbent to be evaluated. Since kedarcidin has potent activity against Gram-positive bacteria [9], the activity of the treated supernatant fluid against *Bacillus subtilus* was determined and used as the criterion for evaluation of the loading capacity of the adsorbents. The results are summarized in Table 1. QMA, DE53 and Trisacryl had relatively small loading capacities. DEAE Sephadex, QAE Sephadex and DE23 all demonstrated practical loading capacities.

It is important to use a resin that has large liquid:solid ratio in the large scale batch adsorption–desorption process. Both DEAE-Sephadex and QAE-Sephadex showed a very small liquid:solid volume ratio because of swelling property when they were hydrated. DE23 has a good loading capacity, a large liquid:solid ratio and relatively low cost. Further testing by stirring DE23 resin in the culture supernatant fluid for 30 min demonstrated that 25 g DE23 per liter of culture supernatant fluid was adequate to bind kedarcidin from the supernatant fluid to the resin. Based upon these evaluations, DE23 was selected as the anion exchange resin for the isolation of kedarcidin from the 1000-L fermentor cultures.

TABLE 1

Evaluation of the loading capacity of anion-exchange resins for adsorption of kedarcidin from culture medium of strain ATCC 53650

Type of adsorbent[1]	Volume loaded[2]	% Activity detected in the last fraction[3]
Trisacryl	3	85
QMA	4	96
DE53	4	76
QAE Sephadex	12	83
DEAE Sephadex	15	75
DE23	15	71

[1] One gram of each adsorbent was used.

[2] One milliliter of supernatant medium from a 6-day-old culture of actinomycete strain ATCC 53650 was sequentially and cumulatively added to each adsorbent. The biological activity of the treated supernatant fluid was then determined.

[3] The biological activity of the treated supernatant fluid was determined by *Bacillus subtilus* disc assay. The activity of the treated supernatant fluid was then compared to that of the untreated supernatant fluid.

Semi-purification of kedarcidin from a 1000-L fermentor

The downstream processing of kedarcidin from a 1000-L fermentor is summarized in Fig. 1. The 650-L fermentation broth was filtered using a filter press and the mycelium was discarded. The pH of the filtrate was adjusted to 5.6 with 0.5 M HCl. The purpose of adjusting the pH of the filtrate to 5.6 was to lower the binding of the less acidic proteins to DE23 resin. This pH adjustment also reduced binding of unwanted black pigments to the resin. These black pigments interfere with the purification of kedarcidin because they elute very closely with kedarcidin in the gel filtration and ion-exchange chromatographies. The filtrate was added to 16 kg DE23 resin previously equilibrated with 0.05 M sodium acetate buffer (pH 5.6) and stirred for 30 min. The preparation was then fed to a perforated bowl basket centrifuge (Bock model 755) for separation at 1100 × *g*. The DE23 resin retained within the centrifuge bowl was washed in situ with 760 L of 0.05 M sodium acetate buffer (pH 5.6) while the filtrate was discarded. The washed DE23 resin was subsequently removed from the centrifuge bowl and batch-eluted by mixing it with 228 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.5 M NaCl for 30 min. The spent DE23 resin was set aside for regeneration (Fig. 3). The eluate was dialyzed against deionized water at 5 °C using an Amicon hollow fiber ultrafiltration unit equipped with a 10 K filter (model H53PM10) in a cold room (5 °C) to remove the excess NaCl generated from the above elution procedure. After the conductance of the dialysate was reduced to 500 μmhos, the Amicon ultrafiltration unit was changed to ultrafiltration mode and the kedarcidin solution was concentrated to about 12 L. The concentrated kedarcidin solution was then lyophilized in a Virtis lyophilizer (model 25SRC) to yield 229 g kedarcidin solid.

The DE23 resin recovered from the above process was regenerated as shown in Fig. 3. The regenerated DE23 was then used for the isolation of kedarcidin from the second 1000-L fermentor culture. The capacity of the regenerated DE23 resin was as good as the fresh material since 204 g of lyophilized kedarcidin was recovered from the second fermentor culture using the regenerated DE23 resin.

The purity of lyophilized kedarcidin sample was determined by SDS gel electrophoresis. Staining the gel with Coomassie blue followed by densitometer tracing indicated that the major Coomassie staining band (MW 12 000), corresponding to the authentic kedarcidin standard, com-

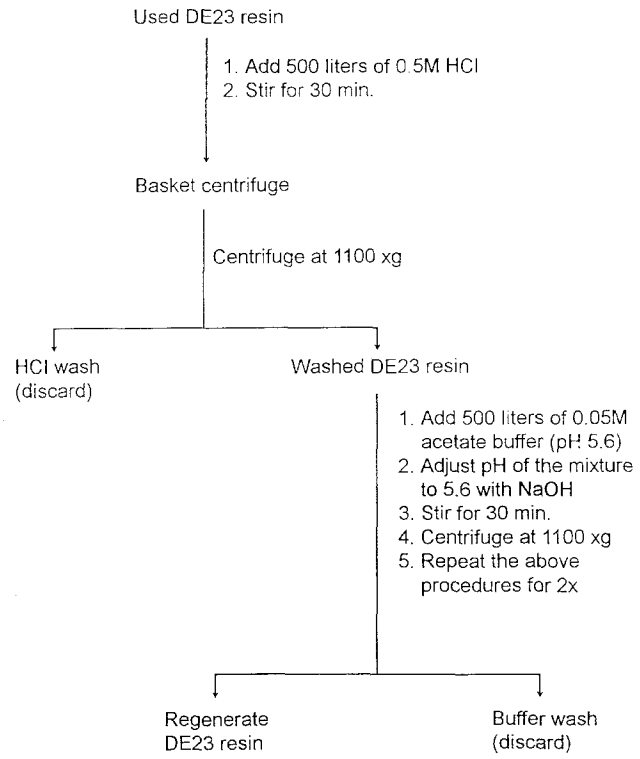

Fig. 3. Regeneration of DE23 resin.

prised 70% of the total protein. Thus, the downstream processing protocol involving anion exchange resin and ultrafiltration is an efficient and convenient method for concentrating kedarcidin from the 1000-L fermentor scale. A 65% recovery yield was achieved. The lyophilized kedarcidin sample is suitable for further purification at a laboratory scale.

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