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Improvement of glidobactin A production by *Polyangium brachysporum*

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SUMMARY

Glidobactins A, B and C are lipopeptide antitumor antibiotics produced by the gliding bacterium *Polyangium brachysporum* sp. nov. No. K481-B101. The production of glidobactin A was examined in shake flasks and laboratory fermentors. Medium screening and optimization led to approximately five fold increases in glidobactin A titers in shake flasks and a ten fold increase in titers in 40-l batch fermentations. Utilization of a stepped glucose feeding protocol resulted in glidobactin A titers of 1860 μg/ml after 144 h of fermentation.

INTRODUCTION

Glidobactins are novel types of antifungal and antitumor antibiotics that are produced by the gliding bacterium *Polyangium brachysporum* sp. nov. K481-B101 (ATCC 53080). Glidobactins are lipopeptides with structures that have been reported previously [4,8]. Glidobactins A, B, and C are normally produced by the microorganism in complex fermentation media. Glidobactins D, E, F, G and H have also been described [7]. The glidobactins are recovered from the fermentation broth by extraction with organic solvents and are purified by a series of chromatographic procedures [5]. Glidobactins have antibiotic activity against fungi, in vitro cytotoxicity against B16 and HCT-116 tumor cell lines, cause inhibition of in vitro DNA and RNA biosynthesis, and have antitumor activity against P388 leukemia, L1210 leukemia, and B16 melanoma in mice [5].

Initial fermentation studies [5] yielded glidobactin A titers of about 150 μg/ml. Subsequent medium improvements [3] boosted the total glidobactin production to about 600 μg/ml for all three components. The ratio of components B and C which are normally present as minor components in the glidobactin complex was increased through the addition of oils and fatty acids to the medium [3]. Numata et al. [3] were able to show significant increases in levels of glidobactin A when methyl palmitoleate ($C_{16:1}$) was added to the base medium. They postulated that glidobactins were synthesized by incorporating the nearly intact form of higher fatty acids into the acyl moiety through β-oxidation. The chemical addition of various fatty acid side chains has been reported [6].

This report expands upon earlier fermentation improvement efforts and describes conditions for production of glidobactin A in laboratory fermentors using batch and fed-batch techniques.

MATERIALS AND METHODS

Microorganism. The glidobactin producing microorganism was the gliding bacterium *Polyangium brachysporum* sp. nov. K481-B101 (ATCC 53080) [5]. Frozen preparations in 10% sucrose were maintained at −70 °C for use as working stocks.

Fermentation. The seed medium, 132 [2,4], consisted of corn starch 2%, Nutrisoy flour 3%, $MgSO_4 \cdot 7H_2O$ 0.3%, and $CaCO_3$ 1%. Seed cultures of 100 mls in 500 ml flasks were incubated for 72 h with shaking at 250 rpm and 28 °C.

Several fermentation media were employed throughout the experimentation. Fr-10-1 [2] was composed of soluble starch 2%, beet molasses 1%, soybean meal 1%, and $CaCO_3$ 0.5%. H173 was composed of corn starch 5%, linseed meal 2.5%, $(NH_4)_2SO_4$ 0.1%, NaCl 0.3%, and $CaCo_3$ 0.6%. H189 had the same composition as H173 with the exception that the corn starch was replaced with 4% cerelose (glucose monohydrate, Corn Products Co.).

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Feeding experiments in 2-l fermentors used the H189 base medium with an initial cerelose concentration of 0.5%. Cerelose feeds were initiated between 10 and 12 h post-inoculation.

Fermentor systems. Four 2-l fermentors were used for studies in small lab fermentors. The fermentors were coupled to a personal computer for data logging. The computer system monitored pH, vessel temperature, dissolved oxygen, agitation speed, off-gas carbon dioxide, and nutrient addition pump rate for each fermentor. For batch studies, 1500 ml of medium were used, and for fed-batch studies, an initial volume of 1200 ml of medium was used. About 400 mls of a 40% cerelose solution were added during the cycle with computer controlled variable speed pumps. Fermentor conditions were 28 °C, 1.5 l/min air flow, and 700 rpm.

Batch fermentations in 70-l fermentors were done with an initial volume of 40 l, 5 psi back pressure, 35 l/min airflow, 300 rpm and 28 °C.

Extraction of glidobactins. Glidobactins were extracted from whole broth samples (2 to 3 ml) with an equal volume of *n*-butanol for 1 h at room temperature while rotating the capped sample tubes. The butanol extract was assayed directly or diluted with *n*-butanol for HPLC assay.

Assays. Packed cell volume was measured following centrifugation of 5 to 10 ml of culture broth at 3000 rpm for 10 min. Glucose was measured using a glucose oxidase reaction (Method 510, Sigma Chemical Co., St. Louis, MO). Total carbohydrates were assayed by the phenol method using sulfuric acid [1].

Glidobactins were assayed by reverse phase HPLC. The column (4.6 × 100 mm) was packed with 5 μm Hypersil ODS. The mobile phase was 75% methanol and 25% water acidified with H_3PO_4 (0.1%) and the solvent flow rate was 2 ml/min. Glidobactins were detected at 254 nm with a retention time of 1.4 min (glidobactin A), 1.8 min (B), and 2.50 min (C). Glidobactin A concentrations of 200 to 1000 μg/ml were used to prepare a standard line for calculation of titers.

RESULTS

Shake flask optimization

Initial fermentations were done in medium FR-10-1 which was used by Numata et al. [3] who reported glidobactin A titers of 200–300 μg/ml. The FR-10-1 medium yielded about 300–400 μg/ml in our laboratory. Subsequent screens of a number of different media showed that H173 gave superior titers when compared with FR-10-1. A series of experiments with the carbohydrate source in H173 led to medium H189. This medium replaced the 5% corn starch in H173 with 4% cerelose.

TABLE 1

Glidobactin A titers of shake flask fermentations in three media

Medium	Glidobactin A Titer (μg/ml)		
	Day 3	Day 4	Day 5
FR-10-1	376	431	353
H173	825	874	820
H189	1197	1284	1308

Titers for 3, 4, and 5 days of fermentation in the different media are shown in Table 1.

Batch fermentations

Preliminary fermentations in 40-l tanks using FR-10-1 medium yielded approximately 150 μg/ml of gildobactin A after 144 h (Fig. 1). The pH in the tank went from 7.9 at inoculation to 7.6 at 48 h and then it rose to 7.8 by 120 h. The titer peaked at 96 h and the total carbohydrate utilization rate slowed at the same time. When H173 was run

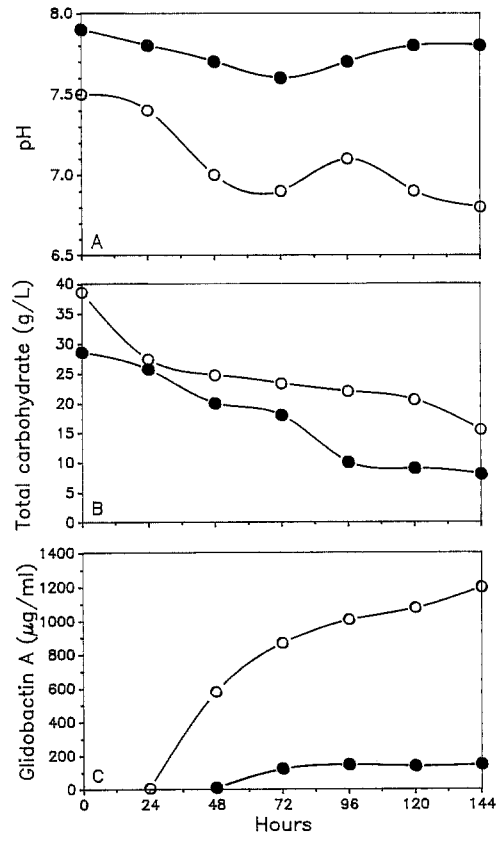

Fig. 1. Fermentation profiles for pH (A), total carbohydrate (B), and glidobactin A titers (C) in 40-l fermentors. o, medium H173, •, medium FR-10-1.

TABLE 2

pH and glidobactin A titers at different feed rates in 2-l fermentors

Feed rate (% glucose/h)	pH after 120 h	Glidobactin A Titer (μg/ml) after 120 h
0.03	7.4	808
0.07	7.2	1206
0.09	6.8	817
Stepped[a]	6.7	1680

[a] See Fig. 2 for feed rates.

in 40-l tanks (Fig. 1) the fermentation yielded about 1200 μg/ml at 144 h. In the H173 medium the pH dropped from 7.5 to 7.0 by 72 h and remained between 7.0 and 7.3 throughout the remainder of the fermentation cycle. Carbohydrate utilization was rapid during the first 24 h and was slower from 24 to 120 h. Carbohydrate utilization rate in H173 was approximately 0.046%/h from 0 to 24 h and 0.008%/h for 24 to 120 h. In FR-10-1 the rates were 0.019%/h for 0 to 96 h and 0.004%/h for 96 to 144 h.

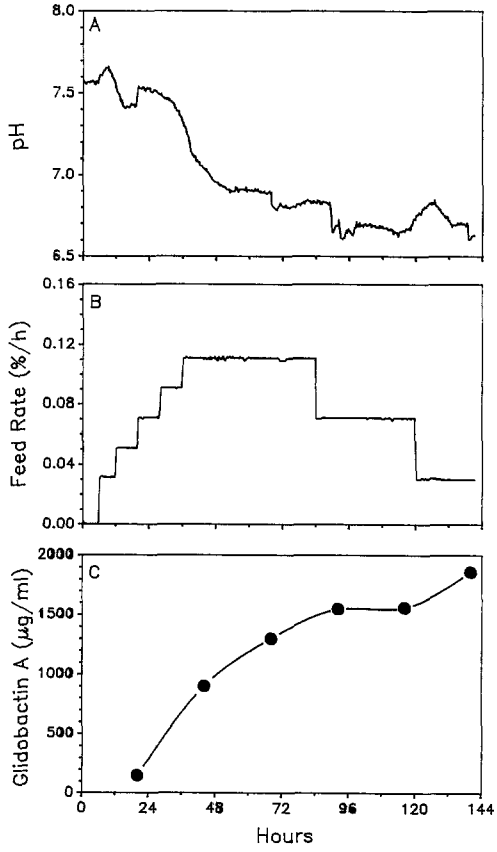

Fig. 2. Fermentation profile for pH (A), feed rate (B), and glidobactin A titer (C) using a stepped feed rate in 2-l fermentors. The base medium was H189 with 0.5% initial cerelose.

Batch fermentations showed pH increases at the same time as the cessation of glidobactin productivity and the limitation of glucose. Fed-batch fermentations with cerelose addition were run at various feed rates as shown in Table 2. Feeds were initiated at 12 h and continued until 120 h into the run. The best constant feed rate was at about 0.07%/h which yielded 1200 μg/ml by 120 h. When a stepped feed rate was used (Fig. 2) significantly higher results were obtained.

DISCUSSION

Gliding bacteria have become an important new source of biologically active molecules [2,9]. *Polyangium* has previously been shown to produce the antifungal compound ambruticin [10]. The glidobactin class of molecules and conditions for their production have been described [3,5]. The organism is readily cultivated in complex media having glucose or starch as the primary carbon and energy source.

The organism did not show significant repression by up to 4% cerelose in the shake flask medium. Cerelose concentrations of over 5% were detrimental to the production of glidobactin A. The FR-10-1 medium used by Numata et al. [3] did not support as high a glidobactin productivity as H173 or H189 described in this report. Both of these media had significantly higher levels of readily available carbohydrate.

Batch fermentations in 40-l tanks with FR-10-1 medium resulted in significant titer losses when compared with shake flasks using the same medium. The high broth pH in the tanks may be responsible for the low titers. Medium H173 supported higher final glidobactin titers in tanks than in shake flasks. The pH profile shows lower pH values than with FR-10-1.

Fed-batch fermentations in 2-l fermentors with the glidobactin culture resulted in higher titers and lower viscosity than with starch-based media such as H173. The optimal glucose feed rate of 0.07%/h was higher than expected based on the utilization of carbohydrate in batch fermentations. The use of a stepped feed rate provided glucose at a slower initial rate, but maintained an excess of glucose in the medium late in the fermentation cycle. Further balancing of nutrients in the fermentor along with the use of the stepped feed could result in higher titers of glidobactin A. The addition of methyl palmitoleate [3] into the fermentor during the fermentation cycle might also result in significant yield increases.

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