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Physostigmine production by *Streptomyces griseofuscus* NRRL 5324: process development and scale-up studies

M Chartrain, L Katz, C Taylor, J Zhang, T Brix, P Salmon and R Greasham

Merck Research Laboratories, RY80Y-105, PO Box 2000, Rahway, NJ 07065, USA

A reliable and scalable fermentation process was developed for production of the acetylcholine esterase inhibitor physostigmine employing *Streptomyces griseofuscus* NRRL 5324. Initial fermentation in small-scale bioreactors reached physostigmine levels of approximately 60 mg L^{-1} after 139 h. Optimization of both process operating parameters and production medium composition rapidly yielded a seven-fold increase in physostigmine titer. The scaled up process routinely produced physostigmine titers of approximately 400 mg L^{-1} during a fermentation cycle of 180 h, and supported the rapid production of large amounts of physostigmine. A physostigmine production of 500 mg L^{-1} representing an eight-fold improvement over the original performance, was achieved using a glucose/ammonium fed-batch process.

Keywords: fermentation; scale up; secondary metabolite; physostigmine

Introduction

Physostigmine, an indole alkaloid found in the seeds of the West African bean *Physostigma venenosum* is a powerful inhibitor of the key neurotransmission enzyme acetylcholine esterase [4]. Several clinical studies report a noticeable improvement in the short term memory of Alzheimer's disease patients receiving either physostigmine or one of its derivatives [1,4,8,11]. Physostigmine can be extracted from the seeds of *P. venenosum*, synthesized through an elaborate multi-step chemical synthesis [7,10], or produced by submerged cultivation of the actinomycetes *Streptomyces griseofuscus* [3], and *Streptomyces pseudogriseolus* [9].

This publication describes a strategic approach to a rapid and efficient process development and its scale up for the large scale production of physostigmine employing *S. griseofuscus*. A systematic optimization of the process operating parameters coupled with the concurrent reformulation of the production medium achieved a seven-fold increase in physostigmine titer. Process stability and reliability were demonstrated at the 800-L scale during a successful pilot plant campaign that supported the timely production of large amounts of physostigmine.

Materials and methods

Inoculum development

A 250-ml Erlenmeyer flask containing 50 ml of ACP medium [3] was inoculated with a frozen cell suspension (1 ml) of *Streptomyces griseofuscus* (NRRL 5324) and incubated aerobically on a rotary shaker (New Brunswick, Edison, NJ, USA) at 220 rpm (2-inch throw) for 48 h at 29° C. The second stage seed was prepared by inoculating 25 ml of the first stage seed to a 2-L Erlenmeyer flask containing 500 ml of ACP medium. This culture was incubated for 48 h under the conditions described above. The third stage seed was prepared by inoculating 500 ml of the second stage seed to a 23-L bioreactor (Chemap, South Plainfield, NJ, USA) containing 15 L of ACP seed medium. The reactor was operated at 29° C, and aerated with 5 L of air per min. Dissolved oxygen tension (DOT) was maintained above 15% of initial saturation by computer-controlled ramping of the agitation (initial set point of 400 rpm).

Physostigmine production (23-L bioreactors)

A total of 500 ml of a third stage seed was used to inoculate a 23-L fermentor containing 15 L of production medium A which contained (per liter of distilled water): Beef extract (Difco, Detroit, MI, USA), 3 g; NZ amine type E (Sheffield, Norwich, NY, USA), 10 g; cerelose, 15 g; ardamine PH (Champlain, Clifton, NJ, USA), 2.5 g; NaCl, 2.5 g; $CaCO_3$, 5 g. Cerelose was routinely sterilized separately and the pH of the medium was adjusted to pH 7.0 prior to autoclaving it. The bioreactor was sparged with air at a rate of 6 L per min, and was operated at 29° C, pH 7.0 with a back pressure of 0.5 bar. DOT was maintained above 10% of intial saturation by computer-controlled ramping of the agitation (400 rpm initial set point, maximum 700 rpm) and by manual increase of the aeration to a maximum of 18 L of air per min.

Process scale-up

The 800-L fermentor was filled with 550 L of production medium and inoculated with a third-stage seed (3.4%, vol/vol). The bioreactor was sparged with 550 L of air per min, and was operated at 29° C, pH of 6.0 with a back pressure of 1 kg cm^{-2}. DOT was maintained above 35% of initial saturation by computer-controlled ramping of the agitation (initial set point of 150 rpm).

Analytical methods

Biomass was measured by dry cell weight. Glucose concentration in the culture supernatant medium was determined

Correspondence: Michel Chartrain, Merck Research Laboratories, RY80Y-105, PO Box 2000, Rahway, NJ 07065, USA
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with a Beckman Glucose Analyzer Model 2 (Beckman, Fullerton, CA, USA). Physostigmine concentrated in the supernatant medium was determined by high pressure liquid chromatography as follows. A broth sample was centrifuged for 10 min at 1300 × *g* in a table-top centrifuge (model TJ 6, Beckman). The supernatant medium was diluted with mobile phase [water (85%) in 0.001% phosphoric acid, acetonitrile (15%) in 0.001% phosphoric acid], and filtered through a 0.45-mm pore size PVDF filter (Gelman, Ann Arbor, MI, USA). Samples (20 μl) were injected, employing an auto injector (model A1-2, Rainin, Wooburn, MA, USA) onto a Zorbax C8 column (Dupont, Wilmington, DE, USA) equipped with a C-8 Brownlee guard column (Applied Biosystems, San Jose, CA, USA) and eluted at a flow rate of 1.5 ml min^{-1} at room temperature (23° C) using the mobile phase described above, and two high pressure pumps model HP (Rainin). The eluant was continuously monitored at 245 nm using a UV detector model UV-1 (Rainin).

Results and discussion

Synthesis of physostigmine by *Streptomyces griseofuscus* strain NRRL 5324 was initially demonstrated in 2-L bench top fermentors. A maximum physostigmine production of 60.8 mg L^{-1} was achieved after 139 h of cultivation (Figure 1). Time course analyses indicate that cell growth ceased following glucose depletion (approximately 45 h after inoculation) and that physostigmine synthesis was initiated during active biomass synthesis and continued during the stationary phase (Figure 1). Physostigmine production was initiated before glucose was exhausted (Figure 1), suggesting a lack of control by this nutrient.

Process parameters optimization

A four-fold increase in physostigmine production (from 61 to 243 mg L^{-1}) was achieved when the concentrations of the complex nutrients in the production medium were increased four-fold (Table 1). This enhanced performance was supported by an increase in both biomass and specific productivity. The use of 4× strength medium taxed the oxygen transfer capacities of the laboratory bioreactors and

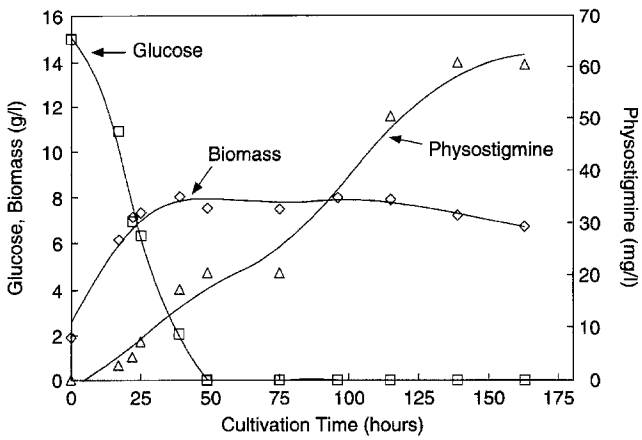

Figure 1 Physostigmine production in a 2-L fermentor. *S. griseofuscus* was cultivated in 1× medium A (see Materials and Methods section for composition) in a 2-L bioreactor

Table 1 Effect of medium strength on physostigmine production[a]

Medium strength[b]	Physostigmine titer (mg L^{-1})	Cell dry weight (g L^{-1})	Specific production (mg g^{-1})
1×	61	8.1	7.5
2×	123	14.5	8.5
3×	200	19.8	10.1
4×	243	22.4	10.8

[a]Experiments were conducted in 23-L bioreactors with dissolved oxygen tension maintained above 10% of initial saturation
[b]Represents increases over the 1× concentrations of production medium A for beef extract, NZ Amine, Ardamine PH, and glucose. Sodium chloride and P 2000 (antifoam) concentrations remained at the original (1×) concentration throughout these experiments

prompted investigation of the sensitivity of physostigmine production to dissolved oxygen tension (DOT) using three strategies. The first strategy employed the delivery of excess oxygen supply (DOT 10%) throughout the entire cultivation cycle, the second achieved oxygen starvation during the growth phase only, and the third used oxygen starvation during the entire fermentation cycle. Typical dissolved oxygen profiles obtained using these strategies are presented in Figure 2. Physostigmine productions of 236 mg L^{-1}, 195 mg L^{-1} and 100 mg L^{-1} were achieved under DOT excess, DOT partial limitation, and total DOT limitation, respectively. The occurrence of maximal physostigmine synthesis under non-limiting dissolved oxygen tension correlates well with what has been observed for the synthesis of several secondary metabolites by other streptomycetes [5,6,12,13]. As suggested by the results of medium strength evaluation studies, it is reasonable to speculate that higher physostigmine production could be achieved when employing a more concentrated production medium combined with the use of highly powered bioreactors and/or pure oxygen sparging.

Table 2 shows that when the pH of the fermentation was controlled in the range of pH 5.75 to pH 6.75, higher physostigmine volumetric production and initial biosynthetic and specific production rates were achieved. Control of pH at 6.0 was selected for the remainder of these process development studies.

Finally, a 1.3-fold increase in physostigmine production (from 255 to 336 mg L^{-1}) was achieved when the temperature of the fermentation was raised from 26° C to 29° C. No further significant increases in physostigmine production were noticed when the temperature was raised above 29° C.

Medium reformulation

Concurrently to the above process optimization studies, a series of experiments was performed to delete the costly and difficult-to-handle beef extract paste from the production medium. After screening various sources of complex nutrients, we selected an acid hydrolyzate of Soy Protein (Champlain, CVP-3S) capable of supporting a physostigmine titer of 105 g L^{-1} (Table 3).

Ingredient concentrations of this new medium were further optimized in shake flasks, employing a two-level fractional factorial design [2]. Computational analyses of

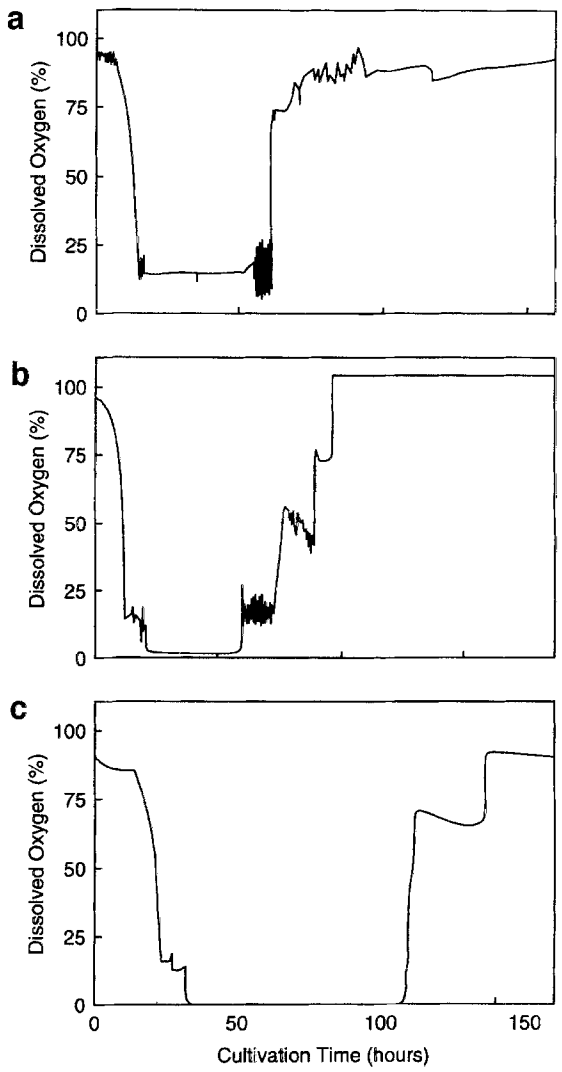

Figure 2 Dissolved oxygen profiles for physostigmine production in 23-L fermentors. (a) DOT was maintained above 10% by increasing the number of impellers from 2 to 3. After 20 h of cultivation, the air flow and the agitation were increased from 8 L to 18 L min^{-1} and from 400 rpm to 650 rpm respectively. (b) Two impellers were used for this fermentation. After 20 h of cultivation, the air flow and the agitation were increased from 8 L to 18 L min^{-1} and from 400 rpm to 650 rpm respectively. (c) Two impellers were used for this fermentation. Air flow was maintained at 8 L min^{-1}, and agitation at 400 rpm

the data showed that cerelose and NZ amine were the two major medium ingredients having a positive effect on physostigmine production. The final run of medium optimization performed in bioreactors employed a central composite experimental design [2] and focused on optimization of both cerelose and NZ amine concentrations. These optimization studies resulted in the formulation of a beef extract-free medium (Soy protein, 4 g; NZ amine type E, 25 g; Cerelose, 65 g; Ardamine PH, 1 g; NaCl, 2.5 g) capable of supporting a physostigmine production of 392 mg L^{-1} after 120 hours of cultivation (Figure 3).

Process scale-up

This laboratory-optimized physostigmine production process was scaled up in a pilot plant (800-L scale) employing highly powered bioreactors. The dissolved oxygen tension

Table 2 Effect of pH on physostigmine production

pH	Physostigmine titer (mg L^{-1})	Initial synthesis rate (mg L^{-1} h^{-1})	Specific production (mg g^{-1})
5.0	0	0	0
5.50	268	3.85	12.9
5.75	320	3.60	13.0
6.0	336	6.25	12.7
6.25	334	5.00	12.0
6.50	325	3.00	12.8
7.0	236	2.98	8.2
7.50	175	1.90	6.2
8.0	61	0.93	2.0

Experiments were conducted in 23-L bioreactors with dissolved oxygen tension maintained above 10% of initial saturation. Temperature was controlled at 29° C

Table 3 Effect of beef extract paste replacement on physostigmine production

Ingredient	Concentration (g L^{-1})	Source	Physostigmine titer (mg L^{-1})
Beef extract	3.0	(Difco)	51
(paste)	9.0		53
Beef extract (dry powder)	3.0	(Lablemco)	95
Peptone	9.0	(Dellac AE80BT)	90
Peptone	9.0	Dellac PP90BT)	81
Primagen	3.0	(Sheffield)	61
Primagen P	9.0	(Sheffield)	62
Hydrolyzed soy protein	3.0	(CVP 3S, Champlain)	105
Hydrolyzed soy protein	3.0	CVP 28SO, Champlain)	92

Experiments were conducted in 2-L shake flasks. Replacement ingredients were tested at the indicated concentration. All other medium component (Medium A) were at the concentration described in the Materials and Methods section.

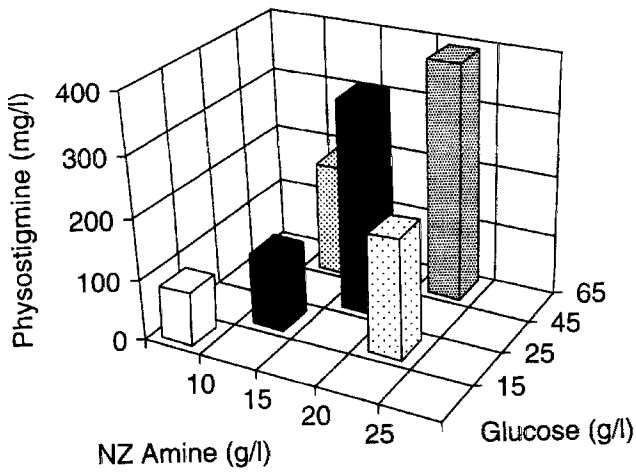

Figure 3 Physostigmine volumetric production. Physostigmine titers presented here are those achieved in production media formulated according to a central composite design aiming at optimizing the amount of glucose and NZ Amine. Concentrations of all other components did not vary and were: Soy Protein 4 g L^{-1}, Ardamine PH 1 g L^{-1}, NaCl 2.5 g L^{-1}.

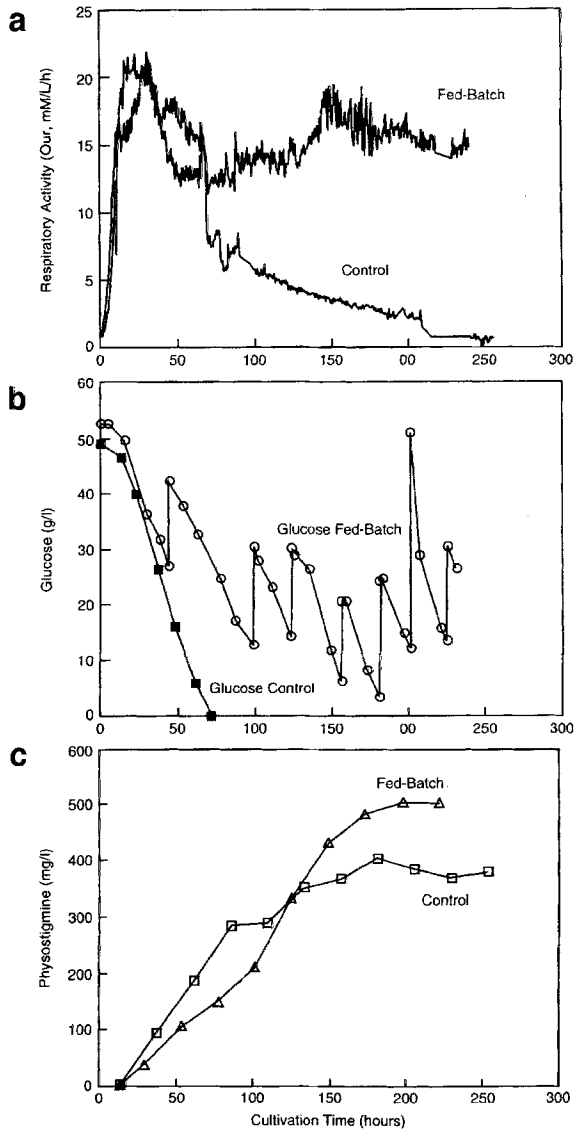

Figure 4 (a) Comparison of respiratory activity (oxygen uptake rate) between the batch process (control) and the fed-batch process. (b) Residual glucose concentration in the batch process (control) and fed-batch process. (c) Physostigmine production in batch process (control) and fed-batch process

was easily maintained at 30% of initial saturation and a physostigmine production level of 400 mg L^{-1} was routinely achieved during fermentation cycles of about 180 hours. Batch cultivation kinetics (Figure 1) showed that physostigmine synthesis was initiated during active biomass production and in the presence of large amounts of glucose. Based on these observations and on the assumption that a prolonged growth phase would support higher physostigmine synthesis, a preliminary glucose/ammonium fed-batch process was tested. Data presented in Figure 4a show that a glucose/ammonium fed-batch supported a steady respiratory activity when compared with the control batch process. As postulated, the presence of glucose did not repress physostigmine (Figure 4b) and under these conditions, a physostigmine production of about 500 mg L^{-1} was achieved (Figure 4c).

While these rapid physostigmine titer improvements were made, a discontinued clinical interest for the derivatives of physostigmine halted these studies. As indicated by preliminary glucose and ammonium feeding experiments which supported physostigmine titers of 500 mg L^{-1}, it is reasonable to speculate that a fully developed fed-batch process, employing slightly increased medium strength, would support even higher physostigmine titers.

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