Journal of Industrial Microbiology, 1 (1986) 39–48
Elsevier
SIM 007

Physiological and genetic factors for process development of cyclosporine fermentations

S.N. Agathos[a,*], J.W. Marshall[a], C. Moraiti[a,**], R. Parekh[b] and C. Madhosingh[b]

[a]*Faculty of Engineering Science, The University of Western Ontario, London, Ontario, Canada N6A 5B9* and [b]*London Research Centre, Agriculture Canada, London, Ontario, Canada N6A 5B7*

Received 15 July 1985
Revised 3 October 1985
Accepted 2 January 1986

Key words: Cyclosporine; Cyclosporin A; *Tolypocladium inflatum*; Process optimization; Strain improvement

SUMMARY

The new immunosuppressive agent cyclosporine (Cyclosporin A, Cy) is the most prominent member of a group of cyclic peptide fungal metabolites (cyclosporins) produced by *Tolypocladium inflatum* in submerged fermentations. In the present study, kinetics and physiology of mycelial growth and Cy production by *T. inflatum* were examined. A new semi-synthetic medium was formulated, consisting of a single carbon/energy source, Bacto-peptone, potassium phosphate and potassium chloride. A wide variety of carbon sources supported growth and Cy production. 3% (w/v) sorbose gave the highest final Cy titer (105.5 mg/l), based on 10-day fermentations. The best specific Cy production was observed with 2% sorbose (14.3 mg Cy/g biomass) followed by 5% *myo*-inositol (13.4 mg Cy/g biomass). A feeding strategy consisting of sequential addition of two carbon sources such as sorbose and maltose was developed in order to reach higher volumetric production. Genetic studies were also conducted, focussing on the development of mutants for increased Cy production and for the synthesis of novel cyclosporins. In the course of these studies, viable protoplasts of *T. inflatum* have been isolated and regenerated.

INTRODUCTION

Cyclosporins are cyclic peptides composed of eleven amino acids (some of them unusual). These cyclic peptides are produced by submerged culturing of aerobic fungi originally identified as strains of *Trichoderma polysporum* [8] but currently classified as belonging to the species *Tolypocladium inflatum* [11]. The major cyclosporin produced in the mycelial fermentations is cyclosporin A, recently renamed cyclosporine (Cy, Fig. 1). After its discovery in the early 1970s by research workers at Sandoz Ltd. (Basel, Switzerland), Cy was shown to be a rather weak, narrow-spectrum antifungal antibiotic [8]. In recent years, however, this fungal metabolite has been the focus of intense interest as an effective

* To whom correspondence should be addressed at (present address): Department of Chemical and Biochemical Engineering, Rutgers University, Busch Campus, P.O. Box 909, Piscataway, NJ 08854, U.S.A.
** Permanent address: National Drug Organization (EOF), Athens, Greece.

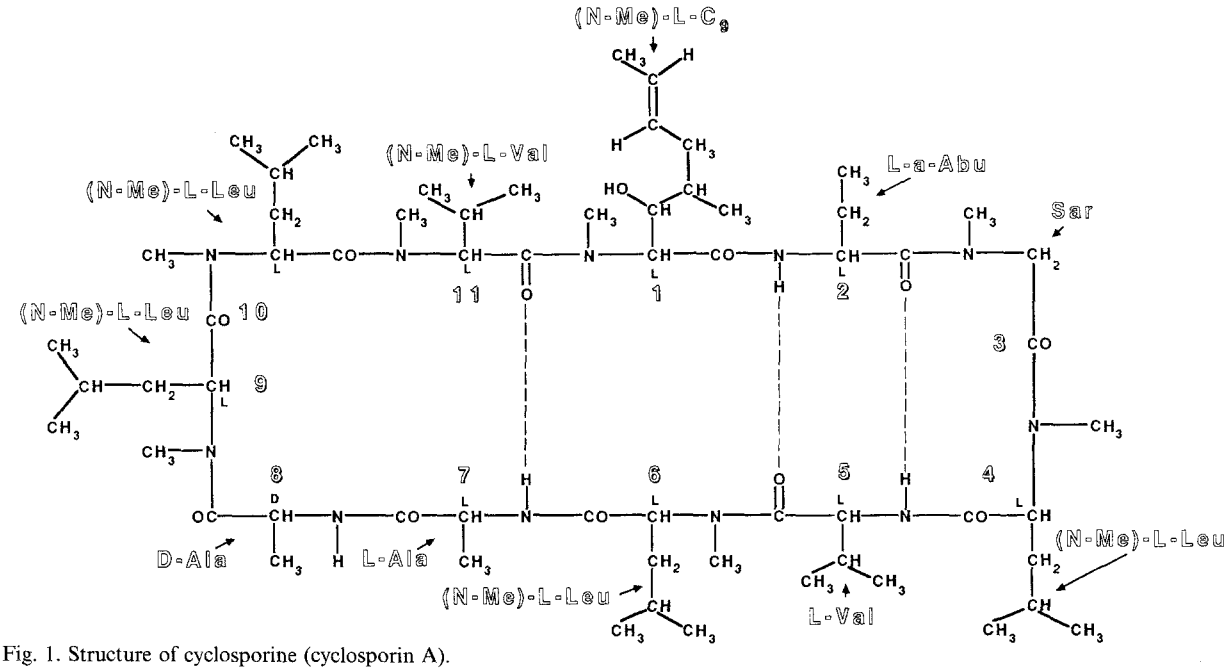

Fig. 1. Structure of cyclosporine (cyclosporin A).

immunosuppressant in organ transplantation [2,3] and also due to its potential in the treatment of autoimmune diseases [14]. In contrast to the numerous clinical studies of Cy, there is only a limited amount of published information on the biology and process engineering of the Cy fermentation.

The production of cyclosporin A and C in submerged fermentation was described by Dreyfuss and co-workers [8] who followed the time course of growth and production of secondary metabolites in shake flasks and stirred-tank fermentors. Kobel and Traber [11] reported on the fermentative production of other, relatively minor cyclosporins, designated B, D, E, F, G and H. These metabolites, as well as cyclosporin C [8], differ from the main product, cyclosporin A, in that they contain different amino acids in positions 1, 2 or 11, whereas those in positions 3 to 10 are the same as in cyclosporin A. Kobel and Traber [11] showed that external supplementation of a defined production medium with the amino acid in position 2 can direct the biosynthesis preferentially towards the formation of the specific cyclosporin characterized by the corresponding amino acid in position 2, i.e., DL-α-aminobutyric acid stimulates preferential production of cyclosporin A and L-threonine stimulates production of cyclosporin C. A report by Foster et al. [9] on the use of immobilized mycelia of *T. inflatum* NRRL 8044 in κ-carageenan beads in a bench-scale airlift bioreactor presents limited time-course data on Cy production, which are compared to free-cell production data from shake flasks.

To date, there has been no systematic examination of carbon and nitrogen nutrition in relation to fungal growth and secondary metabolism in *T. inflatum*. Although the basic morphology of *T. inflatum* has been described by Gams [10], little is known with regard to how it relates to Cy production. In addition, no genetic information on this species has been published.

The present study was undertaken as a starting point in a program aimed at understanding the physiology and secondary metabolism of the Cy fermentation by exploring environmental and genetic avenues for the eventual development of an optimized process.

MATERIALS AND METHODS

All materials used were of analytical grade, with the exception of the solvents which were of HPLC grade.

Organisms. Tolypocladium inflatum (ATCC 34921) was obtained from the American Type Culture Collection, Rockville, MD, *Tolypocladium inflatum* (NRRL 8044) from the Northern Regional Research Labs, U.S. Dept. of Agriculture, Peoria, IL, *Tolypocladium inflatum* (187376) from the Commonwealth Mycological Institute, Kew, U.K. and *Tolypocladium cylindrosporum* was a gift from Dr. G. Barron, The University of Guelph, Guelph, Ontario.

Cultures were maintained on agar slants at 4°C using the medium described by Kobel et al. [12]. This medium consisted of malt extract 2% (w/v), yeast extract 0.4% and agar 2% (pH = 5.7).

Physiological and nutritional investigations

Seed inoculum. Culture suspensions from agar slants were introduced into 250 ml Erlenmeyer flasks that contained 50 ml of MY medium composed of malt extract 2% (w/v), yeast extract 0.4% (w/v) and distilled water to 50 ml, adjusted to pH 5.7. The inoculum was shaken at 200 rpm for 72 h at 27°C.

Shake flask fermentations. 10 ml of seed inoculum (representing 10% (v/v)) were introduced into 500 ml Erlenmeyer flasks that contained 100 ml of SSM medium. This medium consisted of glucose 5% (w/v) or other carbon/energy source as indicated, Bacto-peptone 1.0% (w/v) or other nitrogen source as indicated, KH_2PO_4 0.5% (w/v), KCl 0.25% (w/v) and distilled water to 100 ml, adjusted to pH 5.3. Fermentation was at 27°C, 200 rpm, for 10 days or as indicated.

Biomass determination. Mycelial biomass was estimated by obtaining the dry cell weight of culture filtrates. Sartorius (0.45 μm) filter paper was used and drying was at 105°C for 16 h or to constant weight.

Carbohydrate determination. Carbohydrates were determined by a colorimetric anthrone procedure [15].

Cyclosporin determination

Extraction. 100 ml of butyl acetate were added to the fermentation flasks. The flasks were stoppered and shaken at room temperature (27°C), 200 rpm, for 18 h. The samples were centrifuged at 10 000 *g* for 10 min to separate the organic and aqueous layers. 10 ml of the butyl acetate layer were removed, dried under N_2 gas and re-dissolved in methanol.

HPLC analysis. The mobile phase consisted of acetonitrile/methanol/water (42.5:20:37.5) with a flow rate of 0.8 ml/min. A 15 cm column (2 mm i.d.) packed with 5 μm Spherisorb C_8 was maintained at 72°C and an operating pressure of 1200 lb/in.^{2}. The detector was set at 210 nm and 0.02 attenuation units full scale. 5 μl of sample were injected for analysis. The usual chart speed was 0.25 cm/min. This procedure is essentially the same as that described by Carruthers et al. [4].

Genetic investigations

Antibiotic tolerance testings were performed in MY medium containing 2% Bacto-agar in petri plates. Concentrations of the antibiotics mycostatin, cycloheximide and chloramphenicol up to maximum solubilities of chloramphenicol (38 mM) and mycostatin (8 kunits/ml) in the system and up to 16 mM of cycloheximide were examined. Control plates without the antibiotics were included for comparison. All cultures were incubated at 27°C for periods up to 15 days.

Protoplasts were obtained essentially following the method of Dickinson and Isenberg [7] and modified as described by Madhosingh and Orr [13] using Novozym 234 (Novo Industries, Novo Alle, DK 2880 Bagsvaerd, Denmark) to remove cell walls during 12 to 18 h digestion. For mycelial protoplasts, cultures were prepared on the semi-synthetic maltose medium (SSM) containing 5% (w/v) maltose, whereas conidial protoplasts were prepared from spores developed in the methyl cellulose me-

dium described by Booth [1] as a general method for spore production in fungi. Protoplasts obtained in the above manner regenerated to conidia in a regenerating medium consisting of 0.1 g agar (Difco), 5 g sorbose and 0.32 g EDTA in 50 ml distilled water.

Three mutagens, methyl sulfate, epichlorohydrin and nitrosoguanidine, were used to treat conidia of the isolates in order to develop auxotrophic and antibiotic-resistant mutants. For each mutagen a 50% survival curve based on concentration and time of treatment was determined on each isolate. This concentration was used for subsequent treatments.

RESULTS AND DISCUSSION

Medium development

Defined media are employed, as a rule, to elucidate biosynthetic pathways or regulatory mechanisms. Neither of these two aspects is currently understood in the cyclosporine fermentation. A variety of media has been used in the few available studies to date ranging from complex [8] to synthetic [11]. The latter medium incorporated two distinct carbon sources (sucrose and malic acid at 50 g/l and 8 g/l, respectively), plus a complement of salts, trace elements and vitamins (thiamin, biotin and pyridoxin), whereas nitrogen was provided mainly through adjusting the medium pH to 4.5 with NH_4OH. Our initial trials failed to establish the necessity for all these components. Instead, we developed a simpler, semi-synthetic medium, SSM, consisting of a single carbon/energy source, a complex nitrogen source, potassium chloride and potassium phosphate, after having established that neither ammonium nor nitrate were effective for Cy production by the ATCC strain of *T. inflatum*. In our investigations of carbon/energy and nitrogen

Table 1

Production of cyclosporin A using different carbon sources, based on 10-day fermentations of *Tolypocladium inflatum*

Carbon source (w/v)		Biomass (g/l)	Volumetric production (mg/l)	Specific production (mg/g biomass)
myo-Inositol	(5%)	3.2	47.8	14.9
Cellobiose	(4%)	6.6	80.2	12.2
Galactose	(4%)	7.4	80.4	10.9
Sucrose	(4%)	6.1	46.5	7.6
Citric acid	(5%)	2.0	15.0	7.5
Pyruvic acid	(5%)	1.6	10.7	6.7
Rhamnose	(4%)	0.7	4.7	6.6
Fructose	(4%)	10.7	66.1	6.2
α-D-Glucose	(4%)	9.5	51.4	5.4
Maltose	(5%)	8.5	40.1	4.7
Lactose	(5%)	7.5	31.0	4.1
Sorbose	(5%)	14.0	55.2	3.9
Arabinose	(4%)	5.3	20.0	3.8
Xylose	(4%)	7.9	25.4	3.2
Sorbitol	(4%)	9.7	24.0	2.5
β-D-Glucose	(5%)	9.1	18.2	2.0
Dextrin	(5%)	12.0	22.5	1.9
Mannitol	(5%)	11.6	21.7	1.9
Mannose	(5%)	10.2	10.6	1.0
Glycerol	(5%)	12.4	9.5	0.8
Ribose	(5%)	9.3	6.9	0.7
Sodium acetate	(5%)	0.1	0.0	0.0

Table 2

Production of cyclosporin A using different concentrations of carbon sources, based on 10-day fermentations of *Tolypocladium inflatum*

Carbon source (w/v)		Final pH	Biomass (g/l)	Volumetric production (mg/l)	Specific production (mg/g biomass)
myo-Inositol	(7.5%)	6.1	4.9	42.6	8.7
	(5.0%)	6.1	3.5	47.0	13.4
	(4.0%)	6.1	6.7	40.0	6.0
	(3.0%)	6.4	4.7	42.0	8.9
	(2.0%)	6.5	5.2	32.2	6.2
Maltose	(7.5%)	4.4	13.9	26.7	1.9
	(5.0%)	4.8	11.3	44.9	4.0
	(4.0%)	4.7	10.3	36.5	3.5
	(3.0%)	4.9	8.2	41.2	5.0
	(2.0%)	6.0	6.8	51.0	7.5
Sorbose	(7.5%)	4.7	10.3	104.0	10.1
	(5.0%)	4.5	12.2	56.0	4.6
	(4.0%)	4.8	13.8	43.5	3.2
	(3.0%)	4.8	9.4	105.5	11.2
	(2.0%)	6.0	6.8	97.3	14.3
Fructose	(7.5%)	4.4	12.0	68.6	5.7
	(5.0%)	4.5	10.3	47.8	4.6
	(4.0%)	4.5	10.7	66.1	6.2
	(3.0%)	4.7	11.2	75.0	6.7
	(2.0%)	5.3	7.7	75.7	9.8
Sucrose	(7.5%)	5.0	8.2	51.7	6.3
	(5.0%)	5.2	7.9	41.6	5.3
	(4.0%)	5.1	6.1	46.5	7.6
	(3.0%)	5.3	3.3	37.3	11.3
	(2.0%)	5.3	5.4	34.3	6.4
α-D-Glucose	(7.5%)	4.6	9.8	41.1	4.2
	(5.0%)	4.7	9.5	52.7	5.5
	(4.0%)	4.7	9.5	51.4	5.4
	(3.0%)	4.7	9.7	52.7	5.4
	(2.0%)	5.8	6.0	69.9	11.7

sources, we used the following version of SSM production medium as a standard: glucose 50 g/l, Bacto-peptone 10 g/l, KCl 2.5 g/l and KH_2PO_4 5 g/l. The ATCC strain was used throughout.

Effect of carbon/energy source

An investigation of 22 different carbon/energy sources in SSM medium showed that glucose could be replaced by a broad spectrum of carbon compounds including pentoses, hexoses, disaccharides, polysaccharides and organic acids. Table I depicts mycelial growth and Cy production data obtained with these various carbon sources in order of decreasing specific productivity. Table 2 shows the response of *T. inflatum* to different concentrations of selected carbon sources. Specific Cy productions

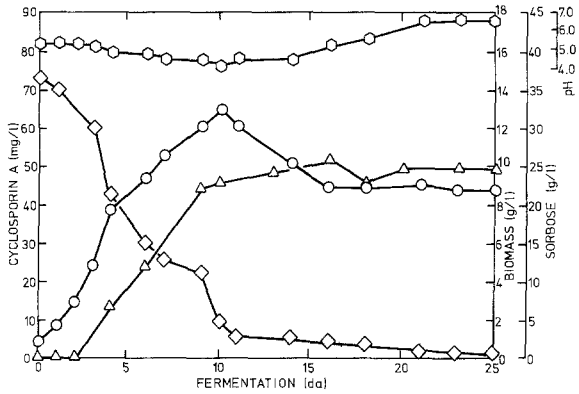

Fig. 2. Sorbose utilization (◇), growth (○), pH (○) cyclosporine production (△) from *T. inflatum* as a function of time.

ranged from zero to approximately 15 mg Cy/g of mycelial biomass. The best specific productivities were achieved with *myo*-inositol (5% w/v) and sorbose (2%), i.e., 13.4 mg Cy/g biomass and 14.3 Cy/g biomass, respectively, while sorbose (3% w/v) showed the highest final titer (volumetric production 105.5 mg CY/l). However, cellobiose, galactose, *myo*-inositol, sucrose, fructose, maltose and α-D-glucose were also favorable C-sources for the volumetric production of Cy, since they contributed to Cy titers in excess of 40 mg/l. By comparison, Dreyfuss et al. [8] using an industrial strain of *T. inflatum* and a semi-synthetic medium containing glucose (40 g/l) as a single C-source, obtained approximately 180 mg Cy/l or 12 mg Cy/g biomass by day 10 of a shake-flask cultivation, and approximately 200 mg Cy/l or 14 mg Cy/g biomass by day 12 of a fermentor cultivation. Kobel and Traber [11] obtained 249 mg Cy/l or 13.1 mg Cy/g biomass in 14-day shake-flask fermentations using an industrial mutant of *T. inflatum* NRRL 8044 and their defined medium supplemented with DL-α-aminobutyric acid. It is evident from our results (Table 1) that C-sources which are highly favored for biomass production by *T. inflatum* do not generally provide the physiological state necessary for optimal secondary metabolite formation. This occurrence is frequently observed in a wide variety of antibiotic fermentations including β-lactams and amino-glucosides [6], and the same trend is apparent in the concentration effect of glucose and maltose upon mycelial biomass and volumetric production (Table 2). Increasing the concentration of C-source does not necessarily increase the productivity of Cy (Table 2). This suggests that each C-source ought to be tested separately as to its optimal concentration for Cy production.

Batch fermentation kinetics

Fig. 2 presents kinetic data from a typical batch fermentation of *T. inflatum* in SSM medium using 4% (w/v) sorbose as the C-source. It is apparent that there is no significant separation of trophophase and idiophase [6], as Cy is detected almost from the beginning of the fermentation. The process is essentially complete by $t = 10$ days; beyond that point there is considerable decrease in mycelial biomass due to extensive hyphae fragmentation and lysis. The product, which is hydrophobic, remains almost completely associated with the mycelium and there is little further production after day 10. The patterns of growth, secondary metabolite production and C-source utilization are in general agreement with the data provided by Dreyfuss et al. [8] for batch Cy fermentations using an industrial strain of *T. inflatum* and a medium containing glucose as the C-source and a combination of $NaNO_3$ and caseinpeptone as the nitrogen source.

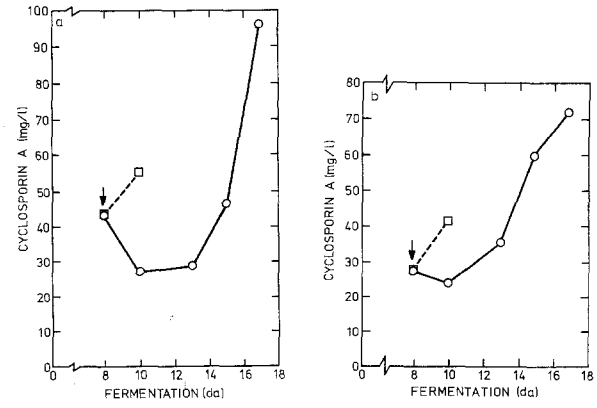

Fig. 3. (a) Volumetric production of cyclosporine using sorbose as a primary carbon source (□) and maltose as a secondary C-source (○). ↓ indicates time of addition of secondary C-source. (b) Volumetric production of cyclosporine using glucose as a primary carbon source (□) and maltose as a secondary source (○). ↓ indicates time of addition of secondary C-source.

In both cases, an initial, somewhat more rapid growth phase is associated with a rapid uptake of the C-source followed by a slower growth phase during which most of the Cy production takes place.

Sequential use of two carbon sources

The addition of a second C-source 8 days after the start of the fermentation in SSM medium initially containing another C-source further stimulated production of Cy. Maltose at 2% (w/v) was used as a second C-source to supplement fermentations that had been initiated with either sorbose or glucose at 4% (w/v) as the primary C-source (see Fig. 3a,b). In the case of glucose as the initial C-source, sequential feeding with maltose brought about a 3-fold increase in the final titer (73 mg Cy/l), whereas in the case of a parallel fermentation started with sorbose, supplementation with maltose at day 8 produced a final titer of almost 100 mg Cy/l which represented more than twice the volumetric production anticipated with 4% sorbose alone. As expected, the use of sorbose as a primary C-source predisposed the culture for higher secondary metabolic activity, since this C-source was found to be superior to glucose for higher volumetric Cy production. Maltose was used as a secondary C-source at a point when the culture of *T. inflatum* was actively producing Cy, since it had been shown before that this C-source was among the more beneficial in ensuring high specific productivities. The second C-source was metabolized to a significant degree during the 2–4-day lag in production following the supplemental feeding (data not shown). Although we have no explanation for the observed decrease in cyclosporine concentration during this lag period in both fermentations (Fig. 3a,b), we speculate that some form of carbon catabolite regulation occurs, during which the Cy levels are partially depleted either through degradation or metabolism in the presence of the second C-source.

The elucidation of this regulatory phenomenon may be important for the eventual optimization of full-scale Cy fermentations. One obvious approach in practice would be a two-stage fermentation using two different C-sources.

Table 3

Production of cyclosporin A using different nitrogen sources, based on 10-day fermentation of *Tolypocladium inflatum*

Nitrogen source (w/v)		Biomass (g/l)	Volumetric production (mg/l)	Specific production (mg/g biomass)
Bactopeptone	(0.5%)	9	18.0	2.0
	(1.0%)	13.2	45.2	3.4
	(1.5%)	12.3	39.0	3.2
	(3.0%)	13.5	39.6	2.9
	(5.0%)	10.4	28.1	2.7
Soytone	(0.5%)	9	1.5	0.16
	(1.0%)	10	3.0	0.33
	(1.5%)	14	5.0	0.36
	(3.0%)	18	12.9	0.72
	(5.0%)	21.2	24.0	1.13
Corn steep liquor	(0.5%)	—	1.2	—
	(1.0%)	—	4.6	—
	(1.5%)	—	6.8	—
	(3.0%)	—	18.3	—
	(5.0%)	—	23.1	—

Effect of nitrogen source

Early on in our investigations with different variations of the published growth media for Cy production [8,11,12] it was important to decide upon the preferred nitrogen source. Inorganic nitrogen sources such as ammonium and nitrate salts did not prove satisfactory for Cy production. Therefore we turned to complex N-sources (Table 3). These different N-sources and concentrations were used as ingredients of the standard SSM medium that contained 5% glucose as the C-source. It can be seen from Table 3 that Bacto-peptone was superior to both soytone and corn steep liquor with respect to both volumetric and specific Cy production. Corn steep liquor proved difficult to handle in conjunction with mycelial mass determination, therefore no indication of biomass is given in this case. 1% (w/v) of Bacto-peptone was adopted as an integral part of SSM medium for all subsequent investigations.

Effect of temperature

In our initial trials of different media and fermentation conditions in shake-flask experiments with *T. inflatum*, we used temperatures between 25 and 30°C, with no significant variations in growth or Cy production. Therefore, in accordance with previous work [8,12] we adopted a temperature of 27°C for most of the studies reported here. However, when we examined the secondary metabolism of *T. inflatum* at a significantly lower temperature of incubation in SSM medium (C-source: 5% w/v maltose) we did observe almost double the final titer in 10 day fermentations over that of a control at 27°C, as follows:

	Temperature (°C)	Cyclosporin A (mg/l)	Cyclosporin C (mg/l)
Control	27	33.6	2.4
	15	57.2	4.0

Although these are only preliminary results, they point to a potentially significant temperature effect for higher Cy production, which may signify that optimum induction or catalytic potency of one or several enzymes involved in its biosynthesis may occur at considerably lower temperatures than those required for growth. This effect may be also related to higher dissolved O_2 (D.O.) concentration at lower temperature.

Preliminary genetic studies

A program of genetic studies was undertaken aimed at the eventual development of mutants and protoplast recombinants with increased yields of cyclosporins and possibly the production of novel metabolites.

(a) Antibiotic tolerance

In the course of initial investigations of mutants with antibiotic resistance markers we studied the susceptibility of four different strains of *Tolypocladium* to chloramphenicol, mycostatin and cycloheximide in solid surface cultures as described. There was no minimal inhibitory concentration for any of the three antibiotics and any of the four strains, *T. inflatum* ATCC 34921, *T. inflatum* NRRL 8044, *T. inflatum* 198376, or *T. cylindrosporum*, up to the highest antibiotic concentration examined. An incubation of 14 days was used for MY plates inoculated with either mycelia or conidia. In the case of chloramphenicol and mycostatin, the tolerance of all four organisms reached 38 mM

Table 4

Numbers of protoplasts obtained from mycelium and conidia of four *Tolypocladium* strains

Organism	Type of cell	Protoplast concentration (number/ml)
T. inflatum ATCC 34921	mycelium	$7.75 \cdot 10^5$
	conidia	$4.35 \cdot 10^6$
T. inflatum NRRL 8044	mycelium	$1.2 \cdot 10^6$
	conidia	$4.8 \cdot 10^6$
T. cylindrosporum	mycelium	$3.2 \cdot 10^6$
	conidia	no conidia found in cellulose medium
T. inflatum 187376	mycelium	$5.05 \cdot 10^6$
	conidia	$6.0 \cdot 10^6$

and 8 kunits/ml, respectively, which corresponds to the limit of their solubility. The highest cycloheximide concentration tested was 16 mM and even at this level the growth of all four *Tolypocladium* strains continued but at very restricted growth rates. The unusually high tolerances to these particular antibiotics are at least twenty times higher than those exhibited by related organisms such as *Fusarium* species (Madhosingh and Orr, unpublished results). These preliminary results would signify a particularly high resistance to permeability which may reflect some unusual features in the membrane structure of *Tolypocladium*. The data suggest further investigations with other antibiotics, possibly in combination with permeabilizing agents such as sodium deoxycholate, in order to do antibiotic resistance studies for genetic manipulation.

(b) Protoplast studies

As a prelude to protoplast fusion experiments we obtained protoplasts from both mycelia and conidia of the four above-mentioned *Tolypocladium* strains and examined their viability and their regenerative potential. The results are shown in Table 4. In general, protoplasting efficiency for these organisms was higher for conidia obtained in cellulose medium. The protoplasts obtained from conidia varied in size, ranging from 1 to 2 μm. Each conidium released one protoplast entity and each protoplast regenerated a single conidium. The protoplasts of the four *Tolypocladium* strains that were liberated by the enzyme digestion of the cell walls averaged approximately 35% regeneration to viable spores and colony forming units. The regenerative treatment resulted in progeny sharing the same morphology [10] and physiology as the original isolates from which the protoplasts were obtained, thus indicating the reliability of the technique for further protoplast hybridization experiments.

(c) Plasmids

Preliminary examination of the cultures by agarose gel electrophoresis [5] has not demonstrated the presence of plasmids in the four *Tolypocladium* isolates.

(d) Mutagenesis

A number of amino acid auxotrophs were isolated. However, these mutants lost their apparent auxotrophy in the second generation. Nevertheless, two mutants were obtained which demonstrated several-fold increase in Cy yield and one producing extremely low amounts of Cy with respect to the stock isolate. These isolates are currently being reexamined for their Cy productivity and stability. Furthermore, these mutants offer significant potential for subsequent hybridization studies.

ACKNOWLEDGMENTS

We express our appreciation of Mr. J.C. Koegler and Dr. D.J. Freeman for performing the chromatographic determinations of the cyclosporins. We thank the Greek Ministry of National Economy for a NATO post-doctoral fellowship to C. Moraiti. This study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through operating grant AO176 to S.N.A. The use of the facilities of Agriculture Canada are acknowledged with gratitude.

REFERENCES

1 Booth, C. 1971. The genus *Fusarium*. Commonwealth Agricultural Bureau, Farnham Royal.

2 Borel, J.F., C. Feurer, C. Magnee and H. Stähelin. 1977. Effect of the new anti-lymphocytic peptide cyclosporin A in animals. Immunology 32: 1017–1025.

3 Canadian Transplant Study Group. 1984. A randomized clinical trial of cyclosporine in cadaveric renal transplantation. N. Engl. J. Med. 309: 809–815.

4 Carruthers, S.G., D.J. Freeman, J.C. Koegler, W. Howson, P.A. Keown, A. Laupacis and C.R. Stiller. 1983. Simplified liquid-chromatographic analysis for cyclosporin A, and comparison with radioimmunoassay. Clin. Chem. 29: 180–183.

5 Davis, R.W., D. Botstein and J.R. Roth. 1980. A manual for genetic engineering. Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

6 Demain, A.L., Y. Aharonowitz and J.-F. Martin. 1983. Metabolic Control of Secondary Metabolic Pathways. In: Biochemistry and Genetics of Commercially Important Antibiotics (Vining, L., ed.), Ch. 3. Addison-Wesley, Reading, MA.

7 Dickinson, D.P. and I. Isenberg. 1982. Preparation of spheroplasts of *Schizosaccharomyces pombe*. J. Gen. Microbiol. 128: 651–654.

8 Dreyfuss, M., E. Härri, H. Hofmann, H. Kobel, W. Pache and H. Tscherter. 1976. Cyclosporin A and C. New Metabolites from *Trichoderma polysporum* (Link ex Pers.) Rifai. Eur. J. Appl. Microbiol. 3: 125–133.

9 Foster, B.C., R.T. Coutts, F.M. Pasutto and J.B. Dosseter. 1983. Production of cyclosporin A by carageenan-immobilized *Tolypocladium inflatum* in an airlift reactor with external loop. Biotechnol. Lett. 5: 693–696.

10 Gams, W. 1971. Tolypocladium. Eine Hyphomycetengattung mit geschwollenen Phialiden. Persoonia 6: 185–191.

11 Kobel, H. and R. Traber. 1982. Directed biosynthesis of cyclosporins. Eur. J. Appl. Microbiol. Biotechnol. 14: 237–240.

12 Kobel, H., H.R. Loosli and R. Voges. 1983. Contribution to knowledge of the biosynthesis of cyclosporin A. Experientia 39: 873–876.

13 Madhosingh, C. and W. Orr. 1985. Zearalenone production in *Fusarium culmorum* after transformation with DNA of *F. graminearum*. Plant Pathol. 34: 402–407.

14 Stiller, C.R., J. Dupré, M. Gent, M.R. Jenner, P.A. Keown, A. Laupacis, R. Martell, N.W. Rodger, B.V. Graffenried and B.M.J. Wolfe. 1984. Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset. Science 223: 1362–1367.

15 Weiner, J. 1978. Determination of total carbohydrates in beer. J. Inst. Brew. 84: 222.