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Application of oxygen vectors to *Claviceps purpurea* cultivation

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Abstract The application of a two-phase fermentation system for the production of ergot peptide alkaloids by *Claviceps purpurea* is described. Perfluorocarbons (PFC) are used as oxygen vectors in *Claviceps* fermentation for the first time. In shake-flask cultivations, the inclusion of PFC in the medium brings about a five-fold increase in the total alkaloid production and a six-fold increase in the pharmaceutically important component, ergotamine. This rise cannot be correlated with the concentration of the added PFC and it is thought that the enhancement is due to a combination of factors, including the influence of PFC. Other oxygen vectors, such as several hydrocarbons, prove to be poor oxygen carriers in our study. Cultivations with PFC in a bioreactor are reproducible, the maximum total alkaloid and ergotamine production being attained on the 11th and 9th days, respectively. The relatively lower increase in the total alkaloid production in the bioreactor as compared to the shake-flasks is attributed to the unequal oxygen availability in the reactor. Processes with PFC offer the operational advantage of a five-fold reduction in aeration rate.

Introduction

Ergot peptide alkaloids produced by *Claviceps purpurea* are important pharmaceuticals. They find clinical applications in treating Parkinson's disease, Alzheimer's disease, migraine, acromegalia, hyperprolactinaemia and other related physiological disorders.

In recent years, a great deal of interest has been focused on the large-scale fermentative production of ergot alkaloids from *C. purpurea* (as reviewed by Kobel and Sanglier 1986; Rehacek and Sajdl 1990; Didek-Brumec et al. 1996, Lohmeyer and Tudzynski 1997; Mukherjee and Menge 2000). A classical problem in submerged cultivation is the optimal supply of oxygen to *C. purpurea*. The very high oxygen demand during the exponential growth phase can be met by increasing the stirrer speed and/or the air supply rate. However, this causes mechanical stress to the sensitive organism and uncontrolled foam formation in the reactor, thus leading to an uneconomical fermentation process. A promising approach to this problem could be the use of an oxygen carrier with a high oxygen solubility, such as hydrocarbons or perfluorocarbons (PFC). Several researchers have applied these substances to enhance the oxygen supply (and oxygen uptake rate) and, as a consequence, to increase the biomass in different culture systems (Lowe et al. 1998; Rols and Goma 1989). Gil'manov et al. 1996 described the use of different hydrocarbons in shake-flask fermentations with *Claviceps* cultures producing clavine alkaloids. For the first time, we report the application of PFC in shake-flask and stirred-tank reactor cultivation of a *Claviceps* strain producing ergot peptide alkaloids. PFC, having the advantages of stability, recoverability and recyclability, are used successfully in cultivations with other microorganisms (Lowe et al. 1998).

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Materials and methods

Organism

C. purpurea 1029 N5 (a mutant of strain 1029) was used in this study. This organism produces more than 500 mg ergot alkaloids l^{-1}, mainly ergotamine and α-ergokryptine and, of the alkaloids produced, 60% are found extracellularly. This strain was originally developed by Prof. U. Keller (Institut für Biochemie und Molekulare Biologie, TU Berlin, Germany) and we obtained it as a gift from Dr. M. Lohmeyer (Institut für Mikrobiologie, Universität Münster, Germany).

Shake-flask cultivations

The following inoculum medium was used: 10% sucrose, 0.05% KH_2PO_4, 1.0% citric acid monohydrate, 0.1% $Ca(NO_3)_2$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.012% KCl, 0.0007% $FeSO_4 \cdot 7H_2O$, 0.0006% $ZnSO_4 \cdot 7H_2O$, 0.00075% nicotinamide, pH 5.2 (with concentrated ammonia). The fungus was grown in 500-ml Erlenmeyer flasks with

80 ml of this medium in a shaking incubator (200 rpm) at 24 °C for 5 days in the dark. An 8-ml sample of the medium was transferred to a new inoculum medium (80 ml) and grown for 2 days. The cells were centrifuged under sterile conditions and a 10% suspension in sterile water was used to inoculate the main production medium (modified T25 medium; Amici et al. 1966). The composition was the same as the inoculum medium, except that the sucrose and citric acid content were 30% and 1.5%, respectively. The incubation conditions were the same as mentioned before.

Four hydrocarbons were tested as the first oxygen vectors. In shake-flasks, 20 ml of hexane, nonane, decane or toluene were added to 80 ml of the production medium. A 1 ml aliquot of the organic phase was fed daily to make up the loss due to evaporation. The cultivations were conducted for up to 13 days and samples were taken at regular intervals.

The commercial PFC, Hostinert 216 (a gift from Hoechst, Frankfurt, Germany), was used in our investigations. In this set of experiments, eight different PFC concentrations were tested (Table 1). The total volume (volume of PFC + volume of medium) in the flasks was always 200 ml. The flasks were incubated for 18–20 days; and 3-ml samples of the medium were taken at regular intervals. During the withdrawal of each sample, a calculated amount of PFC was also taken, so that the ratio of the (medium) aqueous phase:organic phase (PFC) always remained constant during the entire course of fermentation.

Cultivation in the bioreactor

We used a 2-l stirred tank, a stainless steel reactor specially fabricated in our mechanical workshop and coupled to a Biostat B control unit (B. Braun Biotech International, Melsungen, Germany). Data acquisition, process control and monitoring were carried out by the real time integrating software platform developed in our Institute. A 0–30 l min^{-1} mass-flow meter (Mass-flow controller 5851; Brooks, USA) regulated the aeration rate; and the exhaust gas was analysed by the EGAS 2 system (Hartmann & Braun, Frankfurt, Germany). During the cultivation period, the pH was maintained constant at 5.2 and the temperature at 24 °C. pH and dissolved oxygen levels were recorded by electrodes from Mettler-Toledo.

200 ml of the cell suspension were added to 1,800 ml of production medium (2 l total working volume) for the control fermentations. Two fermentations with 20% PFC, one each with either 10% or 40% PFC, were carried out. The stirrer speed and aeration rate were altered when necessary, to maintain a minimum pO_2 level above 30%. Antifoam agent (Desmophen 3900, Bayer, Germany) was added prior to autoclaving.

Off-line analyses

The growth of the fungus was recorded by measuring the dry weight and the extracellular protein content. The samples were filtered and the cell residue was dried at 75 °C until the dry weight was constant. The extracellular protein content was measured by a commercial protein test (DC protein assay; Bio-Rad Laboratories, Hercules, Calif.), which is a variation from the standard Lowry test.

The alkaloid content was measured by the modified Van Urk test (Michelon and Kelleher 1963). The calibration curve was obtained with pure ergotamine tartrate (Sigma-Aldrich, Germany) in the range 5–100 μg ml^{-1}. The mathematical evaluation of the total alkaloid content was done according to Lohmeyer et al. (1990).

It was also tested and confirmed that no alkaloid was dissolved in the PFC phase and all the produced alkaloids remained in the aqueous phase.

To determine the ergotamine and α-ergokryptine content by HPLC, NaCl and liquor ammonia were added to the diluted (1:2) extracellular medium of the samples (5 ml) and extracted three times with 10 ml $CHCl_3$. After evaporation of the solvent, the residue was dissolved in 500 μl acetonitrile. Isocratic HPLC was carried out in an eluent (55% CH_3CN, 45% H_2O, 500 mg $(NH_4)_2CO_3$ l^{-1}) at a flow rate of 1.0 ml min^{-1} at room temperature. The injection volume was 20 μl and the UV detector was set at 320 nm.

Results

Shake-flask cultivations with hydrocarbons

In the course of cultivations with hydrocarbons, hexane and toluene were found to be toxic, because they inhibited growth of the fungus and decreased alkaloid production. The dry cell weight and total alkaloid content in cultivations with nonane and decane were lower than those observed in the reference sample. Thus, these hydrocarbons proved to be poor oxygen vectors in cultivations with *C. purpurea*.

Shake-flask cultivation with PFC

All experiments were performed twice. The mean deviation in all off-line determinations was less than 2%. It was noticed that in flasks with 60–80% PFC, the *C. purpurea* mycelium formed pellets which remained attached to the bottom of the flask. Also, with increasing PFC concentration, the medium showed a change in colour from yellow to brown. Table 1 shows the positive effect of the oxygen vector on biomass formation. There

Table 1 Results of cultivating *Claviceps purpurea* in shake-flasks with different concentrations of the perfluorocarbon (*PFC*) Hostinert 216

PFC concentration (%)	Protein (g l^{-1})		Dry weight (g l^{-1})		Alkaloids (g l^{-1})		Ergotamine (g l^{-1})	
	16 days	20 days	16 days	20 days	16 days	20 days	16 days	20 days
0	5.8	6.4	49.0	54.6	217.0	168.0	6.4	7.7
10	3.8	5.4	49.5	60.4	378.0	800.0	14.9	24.0
20	5.1	5.3	58.9	62.4	797.0	835.0	25.4	28.5
30	5.2	5.7	57.8	70.5	795.0	784.0	23.6	26.7
40	5.7	5.6	58.4	67.6	597.0	906.0	23.8	14.2
50	3.1	3.9	55.3	70.5	795.0	1,244.0	23.5	42.4
60	5.9	6.4	51.1	61.0	686.0	974.0	19.6	28.7
70	6.0	7.4	68.2	44.5	717.0	948.0	22.1	26.4
80	8.4	8.4	62.2	74.0	927.0	889.0	23.0	22.7

was no dependence of cell dry weight on concentration of the added PFC. The extracellular protein concentrations in the flasks containing 10–50% PFC were lower than the reference, while the concentrations in flasks containing 60, 70 or 80% PFC were higher than the reference, the maximum being 8.4 g l^{-1} with 80% PFC. From the data for total alkaloid and ergotamine production, it is difficult to predict an optimal concentration of PFC. Notwithstanding, it can be definitely said that PFC has brought about a dramatic increase in alkaloid production, as compared to the reference. At the lower level (10–30% PFC), 378–835 mg alkaloids l^{-1} and 14.9–28.5 mg ergotamine l^{-1} were produced; and at the higher level (40–80% PFC), 597–1,244 mg alkaloids l^{-1} and 14.2–42.4 mg ergotamine l^{-1} were produced. The next step tested 20% PFC in the reactor, considering that a lower concentration would mean a reduced cost of operation.

Cultivations in the bioreactor

From the results of the shake-flask cultivations described earlier, the concentration of PFC to be used was selected as 20%. The two cultivations with 20% PFC are indicated as PFC 20(1) and PFC 20(2) in Table 2 and Figs. 1, 2, 3. The next process was performed with 10% and was designated PFC 10. The fourth process was tested with a relatively higher concentration of 40% PFC and was indicated as PFC 40. As the reference cultivations were not reproducible, they were described as Ref 1 and Ref 2. All off-line determinations were done in duplicate sets; and the mean deviation was less than 2%. From the operational aspect of the process, it was noticed that, whereas in the reference cultivations the aeration rate had to be increased to 3.5 vvm to maintain a minimum pO_2 level of 25–30%, a dissolved oxygen level of 60% could be maintained by only 0.75 vvm in the fermentations with PFC. The stirrer in every process was run in the range 250–450 rpm. Lowered aeration rate reduced the formation of foam. Figure 1 displays the growth of *C. purpurea* as dry cell weight over time during fermentation with and without PFC. As shown, the growth in all cases was comparable until day 5, after which the pattern differed. A constant biomass was attained after day 13 and the maximum value for media with PFC lay between the two references (59.9 g l^{-1} and 33.7 g l^{-1}). The biomass formation for PFC 20(1) and PFC 20(2) was not repro-

Table 2 Results of *C. purpurea* cultivations in the bioreactor using different concentrations of Hostinert 216. See text for full description of each PFC concentration/formulation. *DW* Dry weight

Parameter	PFC concentration/formulation					
	Ref 1	Ref 2	PFC 20(1)	PFC 20(2)	PFC 10	PFC 40
Hostinert 216 (%)	0.0	0.0	20.0	20.0	10.0	40.0
Max. total alkaloid content (mg l^{-1})	504.0	754.0	681.0	681.0	554.0	554.0
Max. ergotamine content (mg l^{-1})	52.5	134.9	132.8	136.4	152.1	143.8
Max. α-ergokryptine content (mg l^{-1})	12.1	14.0	11.0	13.9	21.5	25.1
Yield of total alkaloids (mg alkaloid g^{-1} DW)	10.6	22.6	23.4	18.3	15.5	19.9
Yield of ergotamine (mg ergotamine g^{-1} DW)	0.9	5.0	4.3	3.8	4.2	4.8
Max. productivity (mg $l^{-1}h^{-1}$)	1.8	2.8	2.9	2.9	2.4	2.4

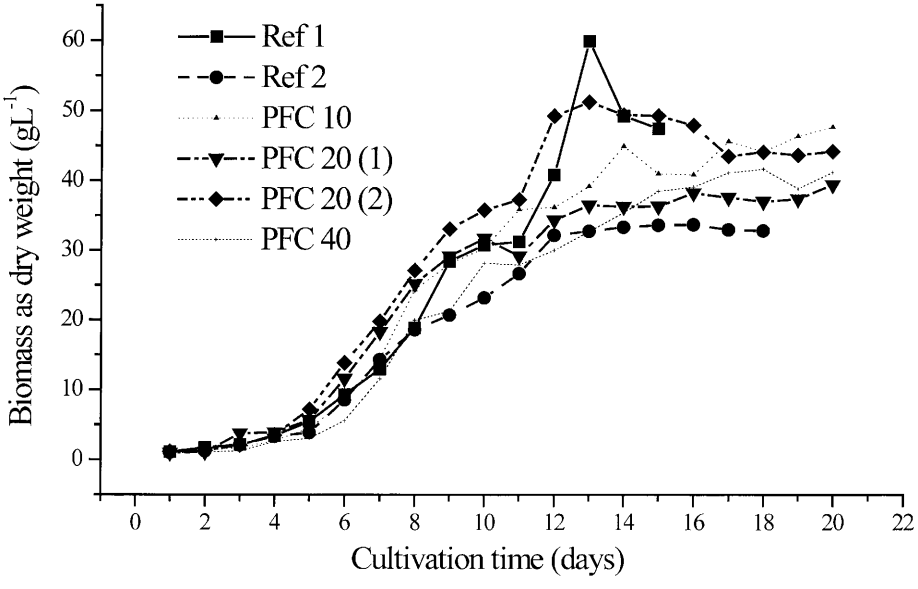

Fig. 1 Biomass as a function of time for the cultivation of *Claviceps purpurea* in a bioreactor, with different concentrations of perfluorocarbon (PFC). The significant results are shown by *thicker lines*. (For a full explanation of abbreviations, see text)

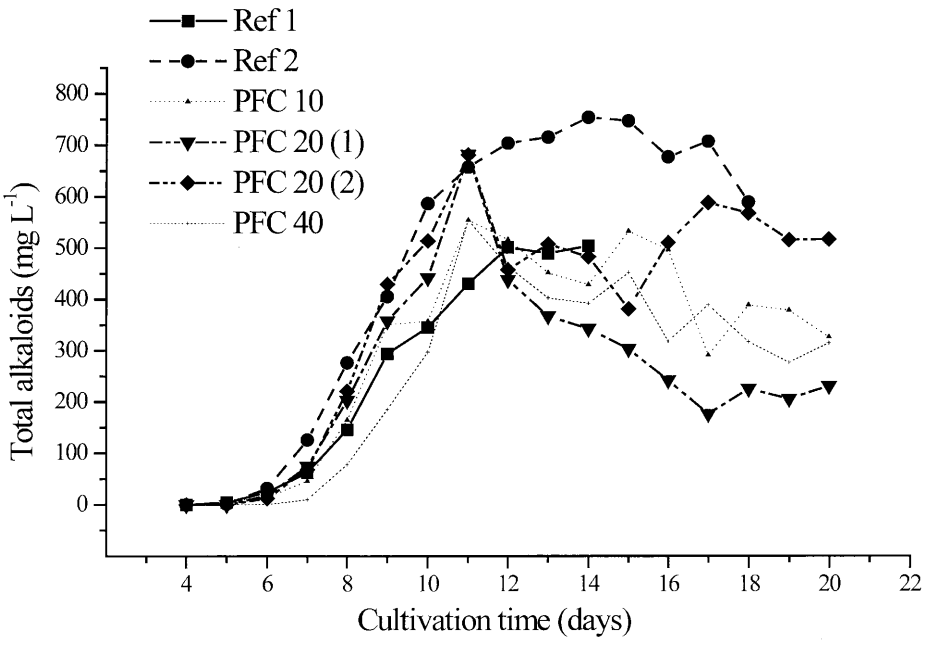

Fig. 2 Total alkaloid concentration as a function of time for cultivations in a bioreactor with different concentrations of PFC. The significant results are shown in *thicker lines*. (For abbreviations, see text)

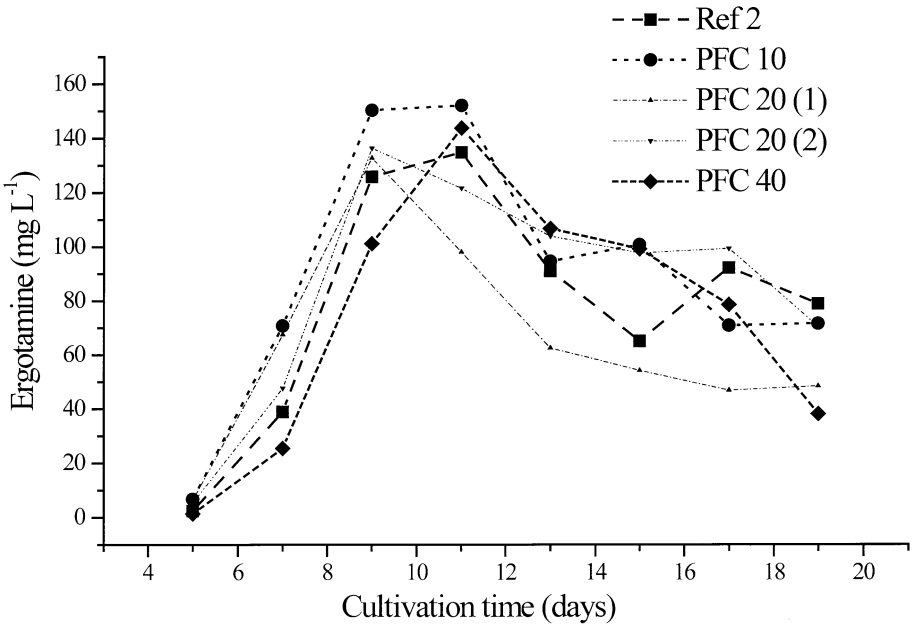

Fig. 3 Ergotamine concentration as a function of time for cultivations in a bioreactor with different concentrations of PFC. The significant results are shown in *thicker lines*. (For abbreviations, see text)

ducible (39.4 g l^{-1} and 51.2 g l^{-1}, respectively); and there was no correlation between biomass formed and PFC added. The extracellular protein (data not shown) was also not correlated. The total alkaloid and ergotamine formation as a function of time are shown in Figs. 2 and 3, respectively. From Fig. 2 it can be concluded that, in cultivations with PFC, the maximum alkaloid production was reached on day 11. PFC 20(1) and PFC 20(2) produced 681 mg total alkaloids l^{-1}, whereas PFC 10 and PFC 40 yielded 554 mg total alkaloids l^{-1}. These values, compared with those for Ref 1, were 35% and 10% higher, respectively; but they were 26% and 10% lower than those for Ref 2, respectively. The two fermentations with 20% PFC were highly reproducible, even to the timing of maximum production. A time course similar to that in Fig. 2 can be seen in Fig. 3; and the maximum ergotamine concentration was also attained within the same time period (days 9–11). The ergotamine concentrations in both PFC 20(1) and PFC 20(2) were nearly the same as in Ref 2, whereas PFC 10 and PFC 40 produced 13% and 7% more ergotamine compared to Ref 2, respectively. The data in Fig. 3 for Ref 1 are incomplete and hence not shown. The required experiments could not be performed with all the samples. However, the determined concentrations were much lower than all the other values shown in the figure.

Table 2 summarizes the results obtained in our bioreactor studies. Of significance is the increase in ergotamine content compared to Ref 1; and the maximum ergokryptine content also shows an enhancement compared to both Ref 1 and Ref 2. However, the results for total alkaloids, ergotamine and maximum productivity are not encouraging.

Discussion

As the solubility of oxygen is higher in organic solvents, a two-phase fermentation system can be used effectively to increase the oxygen transfer rate. Some examples are the use of perfluorodecalin as an oxygen carrier in *Streptomyces* cultivation (Elibol and Mavituna 1995) and the study by Gil'manov et al. (1996), as mentioned earlier. From our study we could establish the PFC Hostiniert 216 as a potential oxygen vector in *C. purpurea* in shake-flask cultivation.

In contrast to the results of Gil'manov et al. (1996), we could not find any positive effect of hydrocarbons when added to *C. purpurea* fermentation media. Elibol and Mavituna (1995) found a dependence of actinorhodin production on the concentration of perfluorodecalin. They reported that production of the antibiotic increased with the addition of up to 50% PFC and then decreased drastically, due to emulsion phase inversion (from PFC in water to water in PFC). However, we did not find any such effect; and alkaloid production by *C. purpurea* in the presence of PFC was influenced by factors other than the concentration of the added oxygen vector.

Within the scope of the present study, it is difficult to explain the large deviations in ergotamine concentrations between the shake-flask experiments containing 40% and 50% PFC. The failure to find an optimal PFC concentration in the shake-flask experiments is an indication that enhancement of alkaloid/ergotamine production is dependent upon factors other than the added PFC. This may be attributed to the complex biochemistry of growth and alkaloid formation in *C. purpurea*.

From a study on the changes in biomass and alkaloid production over time, in a shake-flask with 30% PFC (data not shown in this communication), it was observed that growth became steady after 16 days, compared with 10 days in the reactor; and alkaloid production reached a maximum in the shake-flask also after 16 days, compared with 11 days in the reactor. Thus the reactor offers an advantage as far as the time to formation of the product is concerned. All the PFC cultivations were unwavering, requiring lesser manual attention. The necessity for aeration was significantly reduced, thus leading to less foam formation and lower pressure inside the reactor vessel.

The increase in alkaloid production in the reactor (compared to the reference) was lower than that observed in the shake-flasks. The conditions inside a shake-flask are completely different from those inside a bioreactor; and this is caused by the different ratios of liquid surface:liquid volume, which is responsible for the efficiency of oxygen transfer. For efficient oxygen transfer in the reactor, the PFC phase should be very well dispersed by agitation. This may not have been achieved by the type of impeller used in our reactor system. Even with stirring at 500 rpm, it was seen that the PFC phase (which is more dense and viscous than water) remained in the lower part of the reactor and the upper part was relatively free of the PFC dispersion, thus leading to unequal oxygen transfer in the reactor. The pO_2 electrode used in our system measured the dissolved oxygen concentration in the lower part of the reactor. The presence of another electrode in the upper part could have given a better picture. Also, Pluronik has been used by many researchers (for example Elibol and Mavituna 1995) as an emulsifier to enhance the effects of PFC. But no emulsifiers were added in this investigation. Future work can be done to improve these three aspects, i.e. use of other types of impellers, inclusion of a second dissolved oxygen electrode and addition of Pluronik.

The degradation of ergot alkaloids in the cultivations with PFC can be explained by the presence of degradative enzymes in *C. purpurea*. An example where the product was degraded after its maximum formation has been cited by Rehacek and Sajdl (1990). While such a degradation was not seen in the control fermentation, it was assumed that the degradation mechanism was influenced by the presence of PFC in the medium. It is interesting to note that ergotamine was also degraded in the reference culture without PFC (Fig. 3). The total alkaloid concentration, however, increased during the same time period. This should imply that in both reference cultivations no more ergopeptines but other lysergic acid derivatives or clavine alkaloids were produced after 11 days.

The success or failure of a bioprocess depends upon the formation of a stable product. Although the shake-flask results were promising, the bioreactor studies showed no positive effect of adding PFC to the fermentation medium. Thus the failure to form stable ergot peptide alkaloid molecules in the processes with PFC reveals that these fermentations may not be straightforward; and special attention should be paid to the complex biochemistry of *C. purpurea* when designing a bioprocess with PFC as an oxygen vector.

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