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Lyophilization of Nonsporulating Strains of the Fungus *Claviceps*

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Summary. Maintenance of active strains of microorganisms is one of the fundamental problems for every successful industrial biosynthesis. In this paper, the efficiency of lyophilization for long-term preservation of nonsporulating strains of the fungus *Claviceps*, producing ergot alkaloids in saprophytic cultures, was studied. It was found that lyophilization is a suitable method for the preservation of the tested *Claviceps* strains. All treated strains have retained their vitality and biochemical properties unchanged after three years of storage.

The active strains of certain microorganisms are decisive for various microbiological technical processes. The problem of maintenance of certain strains is thus of great importance. It is generally known (Onions, 1977; Emeis, 1972) that in the course of successive transfers the properties of the microorganisms can change. First of all, the biochemical activity is diminished or totally lost. Highly productive and specialized strains proved particularly unstable. The strains of the fungus *Claviceps*, which produce ergot alkaloids in saprophytic cultures and in chemically defined media, form neither conidia nor spores and exhibit particularly unstable physiological properties.

For this reason, an attempt was made to determine those methods for the conservation of *Claviceps* strains that would retain all their biochemical properties and thus allow the conserved cultures to be available at any time for laboratory and industrial application.

During the last years, the method of lyophilization proved most effective for the conservation of microorganisms. Nevertheless, only a few experiments were reported on the lyophilization of mycelial fungi reproducing only vegetatively. Strains studied in this work (*Claviceps*) belong to this group. For this reason, the lyophilization of the vegetative forms of *C. paspali* and *C. purpurea* was studied in detail.

Materials and Methods

Microorganisms

Claviceps paspali Stev. and Hall. Strain C-10, selected from *C. paspali* ATCC 13892, which does not form conidia and produces lysergic acid derivatives in saprophytic cultures, was used. The cultures were 14 days old.

Claviceps purpurea (Fr) Tul. Strain L-4, selected from *C. purpurea* ATCC 20103, which does not form conidia on chemically defined media and produces mainly ergotamin in saprophytic cultures, was used. The cultures were 14 days old.

Agar media.

For *C. paspali.* Potato agar: 300 g potatoes were boiled, passed through a filter, made up to 1000 ml, 15 g glucose and 20 g agar added, pH adjusted to 6.5.

For *C. purpurea.* Sucrose, 300 g; peptone, 10 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 7 mg; $ZnSO_4 \cdot 7H_2O$, 6 mg; agar, 18 g; distilled water to 1000 ml; pH 6.2.

Protective media.

a) gelatins 5% and sucrose 7%;
b) 2 parts of 60% sucrose and 1 part of skim milk (Difco).

Lyophilizators.

a) 'Lyoboy' of 'Secfroid': lowest temperature in the freezing chamber -70°C; max. vacuum 10^{-2} torr;

b) 'Edwards' Typ 1 PTB, lowest working temperature -50°C; max. vacuum 10^{-2} torr.

Analytical methods.

Mycelial dry weight. A sample of culture broth (100 ml) was filtered through weighed filter paper on a Büchner funnel, washed twice with distilled water, and dried at 85°C to constant weight (Rosazza et al., 1967).

Total alkaloids.

a) Filtrate. Culture filtrate suitably diluted (2 ml) was mixed with van Urk reagent (4 ml) which was prepared as described by Agurell (1966) and the intensity of the blue color determined spectrophotometrically with reference to a standard solution of ergotamine base.

b) Mycelium. Washed mycelium was ground in a mortar and extracted with a mixture of acetone and 4% tartaric acid (1:1) for 3 h. The collected extracts were filtered, properly diluted with water, and used for alkaloid determination as described above.

Alkaloid composition.

a) Thin-Layer Chromatography and Densitometry. The method described by Prošek et al. (1976) was used. Thin-layer chromatography was carried out on commercially available cellulose plates (Merck TLC plates Cellulose—— without fluorescent indicator —— precoated 20 cm X 20 cm, layer thickness 0.10 mm). The plates were activated for 30 min at 105°C and impregnated in a 15% (vol/vol) solution of formamide in acetone. Subsequently, the plates were dried and then heated for 2.5 min at 105°C. The solvent system used consisted of ethylacetate : N-heptane : diethylamine (5:6:0.005).

The samples (0.02%–0.05% chloroform solutions of the alkaloids) were applied with 1 mm^3 Desaga microcaps. The chromatograms were developed in ascending mode in a nonsaturated chromatographic chamber at 4–6°C for about 60 min until the solvent front rose 17 cm from the origin line. Subsequently, they were dried in a stream of cool air. The plates were stored in a dark place for 30 min and then scanned.

Measurements were carried out with the CAMAG TLC densitometer that was combined with a VARIAN A-25 recorder and VARIAN CDS 111 integrator. Ergot alkaloids used as standards were prepared in laboratories of LEK, pharmaceutical and chemical industry, Ljubljana.

b) Liquid Chromatography. A VARIAN 4200 high-performance liquid chromatograph was used. The chromatograph was equipped with a 12.5 cm X 4.2 mm I.D. stainless- steel column filled with 5 μ Spherisorb ODS operated at a flow rate of ca. 80 ml/min at a pressure of about 105 atm. The elution system consisted of water: acetonitrile (60:40, vol/vol) to which 200 mg/l $(NH_4)_2CO_3$ were added. The standard UV detector was operated at 254 nm. A VARIAN A-25 recorder and VARIAN CDS 111 integrator were used.

Preparation of Cultures for Lyophilization

Lyophilization of the microorganisms was performed according to the previously described methods (Raper and Alexander, 1945; Lapage et al., 1970; Cimerman, 1972). Mycelia were homogenized in Waring blendor at 18,000 rpm for 15 sec and suspended in a protective solution of glucose and gelatine or of skim milk and sucrose (Germ. pat. 1969). Samples (0.2 ml) of such concentrated mycelial suspension were transferred into cotton-plugged, sterile glass vials and placed in a cold-chamber at -50°C. After one hour of freezing, the chamber was evacuated to 10^{-2} torr and the temperature maintained at -10°C for 16–20 h during the process.

The tubes with lyophilized cultures were sealed in vacuum and stored in refrigerator at +4°C.

Activity Control

The activity of the lyophilized cultures was determined by fermentation tests carried out before and after lyophilization. A lyophilized sample was rehydrated by adding 0.4 ml sterile physiological solution. After 30 min the rehydrated mycelium was inoculated on agar plates in order to control the survival.

The colonies obtained were used as inoculum for fermentation which was carried out according to the method described by Arcamone et al. (1961) and in the Hungarian patent (1967). Erlenmeyer flasks (500 ml), each containing 100 ml of media were used for incubation. They were shaken at 24°C on a rotary shaker at 250 rpm with a stroke of 50 mm for 14 days. At the end of fermentation, the culture filtrates and mycelia were examined for total alkaloid content and mycelial dry weight. The spectra of alkaloids were determined by liquid chromatography and by densitometry.

Results and Discussion

The efficiency of the lyophilization method was controlled immediately after the process and later yearly during a period of 3 years. The survivial of lyophilized strains of *Claviceps paspali* and *C. purpurea* was satisfactory, yields ranging from 60 to 70%.

To determine the effect of lyophilization on the production characteristics of the strains, data were subjected to statistical analysis.

The initial normal distribution functions of the two strains studied were determined for the alkaloid content and the biomass respectively. The functions were calculated on the basis of 107 samples for *Claviceps paspali* and 44 samples for *C. purpurea*. For the treatment of data after lyophilization, the Student distribution function was applied due to the lower number of samples. Results for the strain of *C. paspali* are summarized in Table 1 and for the strain of *C. purpurea* in Table 2.

Table 1. Total alkaloid content and biomass synthesis by lyophilized cultures of *Claviceps paspali*

Sample		N	$\bar{X}$	s	μ
1	a	108	447.60	151.26	447.60 ± 28.53
	b	108	1.60	0.36	1.60 ± 0.07
2	a	12	458.50	99.03	458.50 ± 65.72
	b	12	1.62	0.26	1.62 ± 0.16
3	a	12	536.17	187.28	536.17 ± 124.28
	b	12	1.91	0.36	1.91 ± 0.23
4	a	10	541.90	176.02	541.90 ± 132.72
	b	10	1.88	0.40	1.80 ± 0.33
5	a	20	618.45	108.71	618.45 ± 52.20
	b	20	1.87	0.32	1.87 ± 0.32

1 – before lyophilization; 2 – immediately after lyophilization; 3 – 1 year after lyophilization; 4 – 2 years after lyophilization; 5 – 3 years after lyophilization. a – total alkaloid content – mg/l; b – dry mycelium weight – g/100 ml; N – number of samples; $\bar{X}$ – mean value of X; s – standard deviation; μ – 1st statistical moment

Table 2. Total alkaloid content and biomass synthesis by lyophilized cultures of *Claviceps purpurea*

Sample		N	$\bar{X}$	s	μ
1	a	44	515.05	154.23	515.05 ± 45.57
	b	44	1.88	0.25	1.88 ± 0.14
2	a	8	510.50	40.23	510.50 ± 35.96
	b	8	1.98	0.16	1.98 ± 0.14
3	a	10	513.80	126.27	513.80 ± 96.21
	b	10	2.07	0.22	2.07 ± 0.16
4	a	15	520.73	140.15	520.73 ± 80.35
	b	15	2.07	0.26	2.07 ± 0.15
5	a	15	523.12	138.16	523.16 ± 35.70
	b	15	2.09	0.24	2.09 ± 0.14

1 – before lyophilization; 2 – immediately after lyophilization; 3 – 1 year after lyophilization; 4 – 2 years after lyophilization; 5 – 3 years after lyophilization. a – total alkaloid content – mg/l; b – dry mycelium weight – g/100 ml; N – number of samples; $\bar{X}$ – mean value of X; s – standard deviation; μ – 1st statistical moment

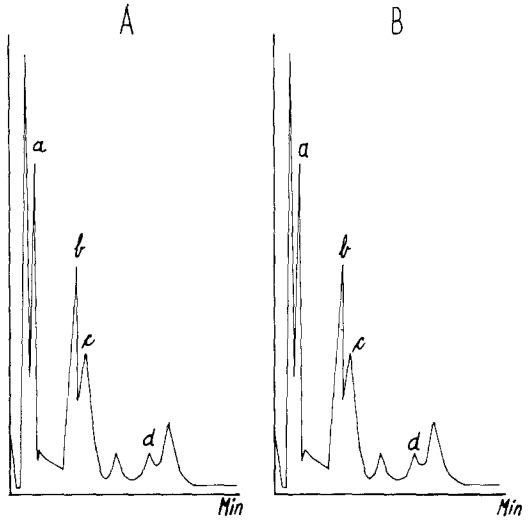

Fig. 1. Liquid chromatogram of lysergic acid derivatives from *Claviceps paspali*. A: before lyophilization; B: 3 years after lyophilization. Peak symbols: a – lysergic acid; b – ergometrine; c – lysergic acid amine; d – isolysergic acid amide

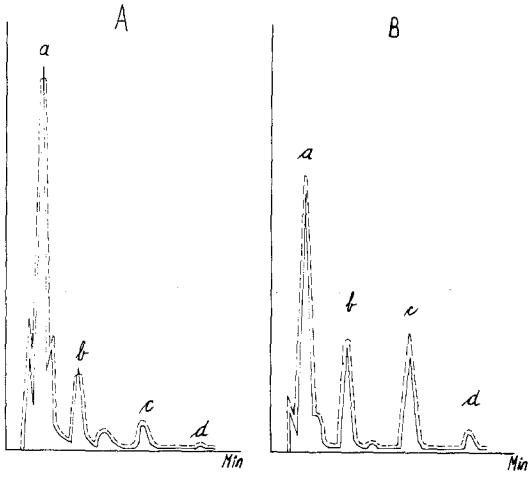

Fig. 2. Densitogram of polypeptide ergot alkaloids from *Claviceps purpurea*. A: in filtrate; B: in mycelium; —— before lyophilization --- 3 years after lyophilization. Peak symbols: a – ergotamine; b – ergotaminine; c – ergokryptine; d – ergokryptinine

Results of the fermentation experiments reveal that neither vitality nor activity was diminished during the process of lyophilization regardless of the protective media used. It was also evident that during several years of storage, the main physiological properties of the strains remained unchanged along with their morphological features. Liquid chromatograms and densitograms revealed that the composition of the alkaloid mixture was unchanged as well (Fig.1 and 2).

Colonies of *C. purpurea* grown on agar were white and cottony, strongly elevated and folded, with craters on the tops and sharp edge. Colonies of *C. paspali* were white, smooth, slightly folded, and convex, with a dark-colored backside. The fermentation broth of lyophilized cultures did not differ from the broth of the initial cultures.

Experimental data from this work indicate that lyophilization is a suitable method for preservation of mycelial forms of the fungus *Claviceps*. Several authors postulated

(Fennel et al., 1950; Hwang, 1966; Mizrahi, Miller, 1968; Homolka, 1976) that this preservation method has certain limitations if applied to vegetative mycelia, since most of them do not survive the treatment.

Our experimental results, on the contrary, indicate that both vitality and biochemical activity of lyophilized cultures remain unchanged even after 3 years storage. In some fermentations, the yield of alkaloids was even better. This could be explained by the fact that only the more vital parts of the mycelia are able to survive lyophilization and are therefore, more active in submerged cultures than initial strains (Onions, 1971; Simmons, 1963).

On the basis of these results, lyophilization proved to be an advantageous method for maintaining *Claviceps* fungi since lyophilized cultures can be easily stored without need for much space and special conditions.

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References

Agurell, S. (1966). Acta Pharm. Suecica **3**, 11–22
Arcamone, F. et al. (1961). Proc. Roy. Soc. **155B**, 26–54
Cimerman, A. (1972). Mikrobiologija **9**, 113–118
Emeis, C.C. (1972). Haltung und Behandlung von technisch wichtigen Mikroorganismen. In: Dechema-Monographien, Vol. 71, H.J. Rehm, pp. 69–78, Frankfurt/Main: Verlag Chemie
Fennel, D.J. et al. (1950). Mycologia **42**, 135–141
Germ. Pat. (1969). 1,806,984
Homolka, L. (1976). Folia Microb. (Prague) **21**, 189–190
Hung. Pat. (1967). 153.739
Hwang, S.W. (1966). Appl. Microbiol. (1966) **14**, 784–788
Lapage, S.P. et al. (1970). Culture Collection and the Preservation of Bacteria. In: Methods in Microbiology Vol. 3A, (J.R. Norris and D.W. Ribbons), pp. 135–228, London, New York: Academic Press
Mizrahi, A., Miller, G. (1968). Appl. Microbiol. **16**, 1100–1101
Onions, A.H.S. (1971). Preservation of Fungi. In: Methods in Microbiology, Vol. 4, (C. Booth), pp. 113–151, London, New York: Academic Press
Prošek, M., Kučan, E., Katić, M., Bano, M. (1976). Chromatographia **9**, 273–276
Prošek, M., Kučan, E., Katić, M., Bano, M. (1976). Chromatographia **9**, 325–327
Rapper, K.B., Alexander, D.F. (1945). Mycologia **37**, 499–525
Rosazza, J.P. et al. (1967). Appl. Microbiol. **15**, 1270–1283
Simmons, E.G. (1963). In: Culture Collections, Perspectives and Problems, pp. 100–110, Toronto: Toronto Press

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