The Maintenance of *Eremothecium ashbyi*

By Z. HOŠT'ÁLEK

Department of General Microbiology, Charles University, Prague 2, Czechoslovakia

SUMMARY: Different methods of maintenance of highly flavinogenic strains of *Eremothecium ashbyi* have been studied.

Storage for 6 months at room temperature or 4° on agar slants either untreated, layered with liquid paraffin or sealed with paraffin wax resulted in considerable loss of flavinogenic capacity. Similar losses also occurred when various strains were transferred regularly at 2-week intervals.

Complete maintenance of flavinogenic activity over 8 months was obtained by freeze-drying cultures, previously frozen at -68°, in maltose or glucose solutions in either water or serum.

In 1935 Guilliermond described *Eremothecium ashbyi*, the parasite of cotton-bolls; on more detailed study this parasite was found to produce a yellow pigment of flavin nature (Guilliermond, Fontaine & Raffy, 1935), which was later identified spectrographically as riboflavin (Raffy, 1938).

The basic study of the physiology of this organism was made by Schopfer (1944). Other authors (e.g. MacLaren, 1952; Dikanskaya, 1953; Hickey, 1953; Goodwin & Pendlington, 1954) followed the nutritional requirements of *Eremothecium ashbyi* and the mechanism of riboflavin synthesis. Recently the results of the older studies are being verified by radioisotopic methods using compounds labelled with ¹⁴C (e.g. McNutt, 1954; Klungsøyr, 1954).

Eremothecium ashbyi was maintained by most authors by passage once a month (Hickey, 1953) or once a week (MacLaren, 1952) on agar slants of similar composition as the liquid media used. Moore & de Becze (1947) consider this the most reliable method of maintaining this organism. By varying the composition of the slants the activity of the strain did not diminish.

Opinions about the maintenance of vitality and biosynthetic activity of *Eremothecium ashbyi*, cultivated on solid media without transfer, vary. Hickey (1953) states that his strain maintained viability on a glucosomaltose agar with yeast extract for 2-3 months. Moore & de Becze (1947) maintained their strain at room temperature for 6 months without loss of biosynthetic activity. When kept in a refrigerator, the strain suffered a considerable loss of riboflavin productivity. Dikanskaya (1954), on the other hand, kept *E. ashbyi* both on solid and on liquid media in the refrigerator for several months, without noting any loss of productivity.

Very little is known about freeze-drying of *Eremothecium ashbyi*. Moore & de Becze (1947) found that *E. ashbyi* on freeze-drying loses its ability to produce riboflavin. In most cases this change was irreversible. Hickey (1953) also states that freeze-drying of *E. ashbyi* was not successful. None of these authors described the method of freeze-drying used, nor did they record results in detail.

* Present address: Antibiotics Research Institute, Roztoky u Prahy, Czechoslovakia.

METHODS

Organism. *Eremothecium ashbyi* originated from the collection of the Department of General Microbiology, Charles University. From a heterogeneous strain with an average riboflavin production of about $170 \mu\text{g./ml.}$, 150 isolates were obtained. Monospore isolates were prepared by cutting out the germinating spore by means of a special pneumatic isolator (Nečásek, Palečková & Tesař, 1953). For the selection of isolates with maximum riboflavin production a screening test was used based on the fluorescence in ultraviolet light of the zones surrounding the colonies (Rytíř, 1955). Three isolates with greatest riboflavin production were used for further experiments.

Cultivation. A liquid medium of the following composition was used: glucose, 1 % (w/v); peptone, 1 % (w/v); yeast autolysate (49 % dry substance), 0.2 % (w/v); distilled water; pH 6.8 before sterilization; sterilized for 20 min. at 1.5 atm. The solid medium contained, in addition to the above ingredients, 1.5 % (w/v) agar.

The organism was cultivated in 500 ml. flasks containing 50 ml. of medium on a reciprocating shaking machine (96 swings/min., amplitude 9.5 cm.) at $28^\circ \pm 1^\circ$.

A standard inoculum was used throughout. It consisted of 0.5 ml. of a 2-day shake culture previously inoculated with a spore suspension (2 ml.) from a 2- or 3-day agar slant. Three-day slants were used when freeze-dried material was examined.

Riboflavin determinations were carried out on 7-day cultures. Differences between three parallel flasks were not more than $\pm 5\%$ of the mean for the three flasks.

Storage on agar slants. The following methods of conservation were used:

(1) *Agar slopes.*

(2) *Agar slopes closed by paraffin wax*—the stopper of the inoculated agar slant was cut off level with the top of the tube and the end of the test tube was then immersed several times in molten paraffin wax.

(3) *Agar slopes, layered by liquid paraffin*—the test tube was slanted so that the end of agar reached approximately to the centre of the test tube; after inoculation it was layered by sterile paraffin oil (of a quality required by Czechoslovak Pharmacopoea) to a level of about 2 cm. above the top of agar.

The cultures were stored either at room temperature, or at 4° . All agar slants were incubated for 6 days at 28° prior to conservation.

Freeze-drying. The method utilizing a system of two test tubes was used (Šourek, 1955). The storage test tubes (7×40 mm.) were inserted into thick-walled test tubes (9×180 mm.) which after desiccation were closed by fusing under vacuum. Agar cultures were used for freeze-drying. The basal solid medium contained 3 % (w/v) agar, in order to prevent contamination of the freeze-dried space by the cultivation-medium during transfer of the culture. The sterilized conservation test tubes were filled by two drops of the freeze-drying medium; the mycelium was well mixed with the inoculating loop by

rubbing against the wall of the test tube and then washed down to the freeze-drying medium. Prior to drying, the material was either frozen for 30 min. in a mixture of glycerol, acetone and solid CO₂, or kept for 30 min. at 4°. It was then dried twice, 4 hr. each, with a 16 hr. long interruption, over P₂O₅ at 0.5 mm. Hg. (During the interruption in drying the material was kept in an evacuated desiccator over P₂O₅.) The freeze-dried samples were stored in darkness at room temperature. For inoculation freeze-dried cultures were shaken up with the basal liquid medium and transferred to an agar slant as a mash. The first control inoculation was made immediately after drying.

Determination of riboflavin. 5 ml. medium were diluted with 40 ml. distilled water, acidified with dilute HCl to pH 4.5 and hydrolysed for 1 hr. at 1 atm. After hydrolysis the sample was made up to 50 ml. and filtered.

Riboflavin in the filtrate was determined polarographically by the method of standard addition (Heyrovský & Zuman, 1953). The determination was carried out in an acetate buffer (Walpole), pH 5.4 in a Heyrovský polarographic cell flushed with nitrogen. Cathodic polarographic curves were recorded from zero potential against the potential of the pool of mercury in the cell. High sensitivity of the galvanometer was used and compensation of the capacity current was therefore applied. E.m.f. 3.8 V. (200 mV./abscissa). The concentration of riboflavin was calculated from the difference between the values of the limiting currents (height of the waves) before and after the addition of a standard solution, according to the formula given in the literature cited above.

RESULTS

Storage on agar slopes

Three monospore isolates (average production: A, 327 µg./ml.; B, 325 µg./ml.; C, 312 µg./ml.) were stored on agar slopes, on agar slopes closed by paraffin wax and on agar slopes layered with liquid paraffin, and kept either at room-temperature or at 4°. They were examined after 2, 4, 6 and 8 months of storage.

Table 1 shows that the flavinogenic capacity of all the stored cultures decreased at approximately the same rate irrespective of the method of storage. After 2 months the decrease was slight but after 8 months the riboflavin levels were the same as those of the heterogeneous strain from which these isolates were originally obtained.

Freeze-drying

The following freeze-drying media were tested: distilled water, saline, dried milk (Difco), whey, 5% (w/v) and 10% (w/v) solutions of glucose, 5% (w/v) and 10% (w/v) solutions of glucose and maltose in serum. Cultures were dried either unfrozen or after freezing at -68°. Two-day non-sporulating cultures and 6-day richly sporulating cultures were stored.

Table 2 shows that the best method of storing freeze-dried *Eremothecium ashbyi* is to lyophilize actively sporulating cultures after first freezing them at -68°, using a 5 or 10% (w/v) glucose solution in water or a 5 or 10% solution

of glucose or maltose in serum. Under these conditions the flavinogenic activity was not significantly reduced after 8 months storage. Distilled water, saline and serum are particularly unsuitable as freeze-drying media.

Table 1. *Production of riboflavin by strongly flavinogenic isolates of Eremothecium ashbyi after storage on agar slants*

Seven-day shake cultures (50 ml. medium in 500 ml. flask) triplicate analysis; temp. 28°; riboflavin in $\mu\text{g./ml.}$

Storage time (months)	Isolate	Method of storage					
		Agar slant Storage temp.		Agar slant closed by paraffin wax Storage temp.		Agar slant covered by liquid paraffin Storage temp.	
		Room temperature	4°	Room temperature	4°	Room temperature	4°
2	A	325	290	329	308	326	319
	B	304	306	327	322	304	346
	C	304	283	337	342	355	277
4	A	240	245	249	263	247	241
	B	246	286	230	243	253	235
	C	228	264	252	226	273	238
6	A	233	216	217	247	206	241
	B	244	215	215	216	213	226
	C	236	223	216	229	232	218
8	A	*	151	184	207	200	205
	B	*	136	194	200	202	196
	C	*	157	210	225	191	188

Original riboflavin levels produced by these isolates were: A, 327 $\mu\text{g./ml.}$; B, 325 $\mu\text{g./ml.}$; C, 312 $\mu\text{g./ml.}$

* The culture did not grow.

Cultures which had been stored for 5 months on agar under paraffin wax, i.e. heterogeneous material with low production were also freeze-dried under optimal conditions. Storage experiments with this material showed a progressive decline in flavinogenic power (Table 3).

Maintenance of strains by regular transfer

The three cultures used in the freeze-drying experiments were also transferred regularly every 2 weeks, in order to compare this with other methods of storage. Riboflavin production was determined at 2, 4 and 6-monthly intervals. It is evident from Table 4, that the flavinogenic capacity of the cultures decreases after twelve transfers to that of the heterogeneous strain.

DISCUSSION

As discussed in the Introduction the general opinion in the literature is that serial transfer of most strains of *Eremothecium ashbyi* at 1- to 3-monthly intervals maintains their riboflavin producing power intact. The present work (Table 4) indicates that using strongly flavinogenic strains this view was not

Table 2. *Riboflavin production by a strongly flavinogenic isolate of Eremothecium ashbyi, after being freeze-dried under different conditions*
Experimental conditions as in Table 1. Riboflavin in $\mu\text{g./ml.}$

		Method of freeze-drying										
		Distil. water	Saline	Dried milk	Whey	Serum	5 % glucose	10 % glucose	5 % glucose in serum	10 % glucose in serum	5 % maltose in serum	10 % maltose in serum
Unsporulated unfrozen culture	Immediately after freeze-drying	205	*	246	239	*	266	248	313	303	284	301
	After 8 months storage	149	*	182	*	*	*	193	219	227	247	214
Unsporulated frozen culture	Immediately after freeze-drying	*	*	289	*	*	307	*	*	*	325	305
	After 8 months storage	*	*	*	156	*	214	*	*	*	254	221
Sporulated unfrozen culture	Immediately after freeze-drying	187	*	201	268	134	248	*	253	204	289	263
	After 8 months storage	*	*	*	223	*	209	231	185	170	201	246
Sporulated frozen culture	Immediately after freeze-drying	*	*	275	303	*	397	321	343	394	465	410
	After 8 months storage	*	*	253	265	*	284	298	348	332	368	348
Riboflavin production prior to freeze-drying 327 $\mu\text{g./ml.}$												
* The cultures did not grow.												

Table 3. *Production of riboflavin by a poorly flavinogenic strain of Eremothecium ashbyi, after being freeze-dried under various conditions*

Experimental conditions as in Table 1. Riboflavin in $\mu\text{g./ml.}$

Time of storage	Culture	Method of freeze-drying			
		5 % maltose	5 % glucose	5 % maltose in serum	5 % glucose in serum
Immediately after freeze-drying	A	218	206	181	188
	B	212	193	200	205
	C	210	198	207	195
3 months	A	176	182	184	183
	B	185	188	192	195
	C	179	179	174	175
6 months	A	190	181	173	180
	B	181	191	173	166
	C	181	169	180	171

Riboflavin production prior to freeze-drying: culture A, 217 $\mu\text{g./ml.}$; B, 215 $\mu\text{g./ml.}$; C, 219 $\mu\text{g./ml.}$

Table 4. *Production of riboflavin ($\mu\text{g./ml.}$) by poorly flavinogenic isolates of Eremothecium ashbyi maintained by regular subculture*

The organisms were subcultured every 2 weeks. Cultures were incubated at 28° for 6 days and then stored at room temperature. Experimental details as Table 1.

Time of subculture (months)	Riboflavin $\mu\text{g./ml.}$		
	Isolate A	Isolate B	Isolate C
0	217	215	219
2	206	212	198
4	199	189	199
6	167	171	170

upheld. Furthermore, agar slants sealed in various ways also begin to lose their flavinogenic activity after 2 months storage, either at room temperature or at 4° (Table 1).

Previous authors (Moore & de Beeze, 1947; Hickey, 1953) have dismissed freeze-drying as unsuitable for conserving *Eremothecium ashbyi* but they did not report in detail the methods used. Conditions have now been defined which allow production of freeze-dried cultures which completely retain their flavinogenic activity over a period of at least 8 months. Any variations in these conditions result in loss of activity.

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