Pharmaceutical Biology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713721640

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Online publication date: 23 December 2009

To cite this Article Johnson, David G. and Gjerstad, Gunnar(1964) 'Variability of Alkaloid Production in Submerged Culture', Pharmaceutical Biology, 4: 1, 577 — 581
To link to this Article: DOI: 10.3109/13880206409178849
URL: http://dx.doi.org/10.3109/13880206409178849

INTERMEDIARY METABOLISM OF ERGOT - X

Variability of Alkaloid Production in Submerged Culture

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In previous publications of this series [1]), we have studied the influence of various physical and chemical factors on the metabolism of authenticated *Claviceps purpurea* (Fries) Tul., officially recognized by most pharmacopoeias or pharmaceutical compendia as the source of the drug Ergot.

Since our rye ergot strain failed to produce alkaloids in laboratory cultures, we acquired several other alleged *Claviceps* strains which were claimed to produce various ergoline alkaloids in saprophytic cultures. We had in the past maintained some skepticism as to their authenticity, and recently Tonolo [2]) seemed to agree by suggesting a reclassification of same into a new genus, *Mothesia.* We felt that our extensive studies of the intermediary metabolism of *Claviceps* might substantiate Tonolo's proposal. We also wanted to check certain information in the literature as to special techniques claimed to be absolutely necessary for alkaloid formation. Gröger *et al.* (1) claimed the following "protocol" as essential: The sclerotia must be inoculated on an agar medium, subsequently transferred to a preculture medium, and finally to a liquid "fermentation" substrate. Any other procedure or route proved unsuccessful. Since a mandatory procedure of this nature would make industrial production difficult, we were vividly interested in substantiating their findings; also those of Brack *et al*'s work (2) which reveal interesting growth and alkaloid production curves. Thirdly, we were aware that most successful workers on said strains employed *tap water* in their media, a procedure which makes it virtually impossible to simulate their experimental conditions in other geographical locations. In this laboratory, distilled water has always been used.

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1) Part IX: Quart. J. Crude Drug Res., IV (1964), 537—540.
2) Newsletter of the American Society of Pharmacognosy *1* (2), 2 (1964) Ed: M. R. Gibson.

EXPERIMENTAL

Materials and Methods —

In our initial studies, we employed 8 strains, the exact botanical or geographical origins of which as well as their precultivation have not been definitely established:

P-47A, obtained through the courtesy of Dr. Egil Ramstad, Purdue University, School of Pharmacy and Pharmacal Sciences, W. Lafayette, Indiana. (3)

W-47A, W-189, W-SD-58, and W-62P were kindly placed at our disposal by Dr. L. R. Brady, School of Pharmacy, University of Washington, Seattle. (1)

ATCC-13892 and ATCC-13893, available from the American Type Culture Collection, Washington, D.C. These cultures were deposited by Istituto Superiore di Sanita, Roma, Italy. (4)

ARE is our original American Rye Ergot, derived from authenticated rye sclerotia of high alkaloid content and which has been studied extensively in our laboratories and by Suhadolnik *et. al.* (5). This variety has not been claimed to produce alkaloids in saprophytic cultures.

We soon realized great difficulties in preparing a basic substrate conducive to the growth of all these, ARE being nutritionally and biochemically least discriminating. This may indicate that the organisms are not closely related genetically. The literature (3) indicates that mannitol appears to be the most favourable carbon source. Numerous media formulations were tested, and initial experiments indicated Medium *A* to be superior. This finding is somewhat surprising; it contains a relatively high concentration of mannitol, which is assumed to be quite different from the nutrition of Ergot's natural habitat.

Medium *A* contained per liter of distilled water (in grams): Mannitol, 100; KH_2PO_4, 1; $MgSO_4$, .3; $FeSO_4$, .0079; $ZnSO_4.7H_2O$, .004; $(NH_4)_2SO_4$, 3.668; Succinic Acid, 5.4. Ammonia was added to render a pH of 5.2. Medium *B* was made from 50 % medium *A*, diluted with 50 % distilled water. Substrate *C* consisted of one part of Medium *A* diluted with 3 parts of water. The media were distributed in 60 ml. portions into 250 ml. Erlenmeyer flasks fitted with cotton plugs and autoclaved at 120° for 15 minutes. At least three parallels were performed for each experiment. During the dark experiments the flasks remained in the dark uninterruptedly, whereas in the 'light condition' they were exposed to laboratory diurnal light variation. Preliminary experiments indicated that alkaloid production was in-

significant during the first three weeks; consequently, the cultures were allowed to grow for 26 days prior to the first assay.
The following reagent was employed routinely for the alkaloid tests: 0,2 % para-dimethyl aminobenzaldehyde (PDAB) in 65 % sulfuric acid, to each 100 ml. of which 1 drop of $FeCl_3$ Test Solution USP was added, the reagent was stored in a refrigerator and renewed weekly.

Assay —

One ml. of the medium was removed aseptically from each flask, and mixed in turn with 2 ml. of PDAB reagent. This mixture was allowed to stand for 30 minutes; a reading of its optical absorption was then determined with a spectrometer (Bausch and Lomb model Spectronic 20) at 590 mμ. After the fourth assay (45 days), the testing was continued only on those two strains (P-47A and W-47A) which had shown significant alkaloid producing ability.
In a supplementary experiment, to test the reproducibility and importance of the Tyler technique only the significant alkaloid producing and the previously untested strains were used, and the cultures were inoculated both from agar slants and from 40-day-old submerged cultures of the first series. The employed procedures were identical to those of the main experiment.

RESULTS AND DISCUSSION

The results, which are average values of triplicate experiments, appear in Tables I and II. Results of parallel tests were in reasonable proximity. Only two of the eight strains produced significant amounts of alkaloids. This was expected from previous experience with these strains. Of primary concern in this experiment was the time factor. It was established that the alkaloid concentration increases markedly, sometimes as much as two-fold beyond a 30-day-growth period, the usual experiment duration.
We consistently found more alkaloids in cultures grown in the dark. It will be noted that the analytical results have been entered as "% Transmittance" rather than as alkaloidal content because we obtained considerable optical density with cultures (ARE) which on paper chromatographical examination failed to reveal ingredients producing a blue colour with PDAB-spray. For a standard curve for alkaloid estimate, see previous work (6). We also noted that several cultures varied in inherent colour which registered a detectable light absorption at 590 mμ (ARE). *We do not, therefore, consider the PDAB-test for ergot alkaloids as unequivocally reliable.*

As a general rule, light absorption increases with time up to a 60-day-cultivation period under our experimental conditions. If a decrease in light transmittance may be interpreted as an increase in alkaloid production, we consider this an important finding, which should be taken advantage of in industrial fermentation. An occasional slight incidental increase might be attributed to changes in inherent colour and analytical inaccuracy. The parallel assays were in reasonable proximity.

It should be noted that a transfer of inoculum from the original submerged culture to another one, *increases* alkaloid content. Further the transfers seem to confirm this finding. We are, therefore, not able to confirm Tyler's report (1), which, if universally applicable, would tend to make industrial production highly inconvenient, if not impracticable.

On the other hand, some isolates did not produce alkaloids in detectable quantities under the experimental conditions.

Table I. INFLUENCE OF VARIOUS FACTORS ON ALKALOID PRODUCTION OF CLAVICEPS STRAINS

Strain	Media	PERCENT TRANSMITTANCE [a] Age of Cultures (days) 26 L[b]	26 D[c]	31 L	31 D	38 L	38 D	45 L	45 D	52 L	52 D	59 L	59 D
P-47A	A	83	80	75	81	47	59	47	48	44	35	43	25
	B	75	82	76	81	67	66	65	49	60	43	64	45
W-47A	A	52	26	53	25	24	8	33	12	43	10	53	7
	B	77	86	76	79	64	69	64	50	65	40	70	43
W-62P	A	99	100	99	98	98	99	98	98				
	B	99	100	98	100	98	100	94	98				
W-189	A	76	83	82	86	73	82	79	86				
	B	96	94	96	96	94	90	92	89				
W-SD58	A	79	92	85	88	78	91	77	89				
	B	97	96	96	94	98	97	96	96				
ATCC 13892	A	97	95	96	96	91	93	82	91				
	B	95	94	98	94	95	94	87	89				
ATCC 13893	A	97	98	93	98	89	93	83	92				
	B	97	100	96	99	90	97	90	91				
ARE	A	68	65	61	63	63	61	62	64				
	B	66	60	62	64	57	62	65	55				

[a] For further explanation, see text.

[b] Grown in intermittent diffuse daylight.

[c] Grown in darkness.

Table II. INFLUENCE OF INOCULATION PROCEDURE ON ALKALOID FORMATION

PERCENT TRANSMITTANCE

Strain	P-47A		W-47A		W-189		W-SD58	
Media	A	B	A	B	A	B	A	B
Source of Inoculum								
Agar Slant	50	70	37	61	80	89	56	83
Liquid Culture	42	63	6	21	85	84	87	87

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ACKNOWLEDGMENT

This investigation was supported, in part, by a Public Health Service Research Grant, No. M-03518-04, from the United States National Institute of Mental Health.