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Direct selection of active *Claviceps* colonies on agar plates

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An agar plate method for the primary screening of high yielding *Claviceps* strains has been developed. It is based on the detection of alkaloids in the colonies in the UV light by adding fluorescent indicators, fluorescein or 2′-7′-dichlorofluorescein, to the solid medium.

Claviceps strains are very efficient producers of medically important ergot alkaloids in submerged cultures. For this purpose, however, high-yielding strains are required which could be obtained by selection and mutagenic treatment. To detect increased titre of mutants screening of large numbers of colonies is essential. The use of shaken liquid cultures for this purpose greatly limits the number of potential mutants that can be tested because of time consuming and laborious work.

Therefore several investigators have devised methods for correlating antibiotic production on agar and in shake flasks (Ditchburn *et al.* 1974, Ishikawa *et al.* 1971, Ball and McGonag 1978) and found that agar plate methods are suitable for primary screening. Similar agar plate methods have been developed also for identifying high acid-yielding microorganisms (James *et al.* 1956, Carls and Hanson 1971) as well as for detecting pectolytic activities of some bacteria (Hankin *et al.* 1971, Hubbel *et al.* 1978).

Since no simple measure except of morphological features for primary screening of *Claviceps* strains was available, we tried to develop such a method which was based on the detection of ergot alkaloids in the colonies during their growth on agar plates. The presence of alkaloids in the colonies could be detected with fluorescent indicators added to the agar medium.

Microorganism: *Claviceps purpurea* ATCC-20103 selected mycelial strain, capable of producing about 1500 mg/liter alkaloids in saprophytic cultures, was used.

Solid medium for plating: SP-agar: It contained g/l: sucrose, 300; casein-peptone, 10; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.007; $ZnSO_4 \cdot 7H_2O$, 0.006; agar-agar, 18; and tap water to volume. Before sterilization the pH of the medium was adjusted to 6.2 with ammonia (German patent, 1973). After sterilization 1.25 ml of 0.5% solution of fluorescein or 2′,7′-dichlorofluorescein in ethanol were added to 250 ml of the medium. This was poured into steril Petri dishes and when the agar had solidified, placed in an incubator (24 °C) and left for some days before inoculating the media.

Screening procedure: Agar plates were inoculated with small fragments of developed colonies or with properly diluted fermentation broth as well as with mycelial suspensions after mutagenic treatment. After inoculation Petri dishes were incubated for 21 days at 24 °C and developing colonies were examined under UV light of 254 nm.

Shake flask fermentations: Fermentation experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml culture medium and being incubated on rotary shaker (Infors RC-100, at 240 rpm with 5 cm stroke) 4—5 days for seed stage fermentation and 14 days for production stage fermentation.

The seed stage medium was modified after Hung. pat. (1967). It contained g/l: sucrose, 100; succinic acid, 10; proflo, 10; $Ca(NO_3)_2$, 1; KH_2PO_4, 0.25; $MgSO_4 \cdot 7\ H_2O$, 0.25; KCl, 0.12; $FeSO_4 \cdot 7\ H_2O$, 0.007; $ZnSO_4 \cdot 7\ H_2O$, 0.006 in 1 l of dest. H_2O.

The production stage medium contained g/l: sucrose, 100; succinic acid, 10; $Ca(NO_3)_2$, 1; KH_2PO_4, 0.3; $MgSO_4 \cdot 7\ H_2O$, 0.25; KCl, 0.12; NaCl, 20; $FeSO_4 \cdot 7\ H_2O$, 0.007; and $ZnSO_4 \cdot 7\ H_2O$, 0.006 in 1 l of dest. H_2O. pH 5.2 in both media was adjusted with ammonia before sterilization.

Analytical methods: Growth of submerged cultures was determined by mycelial dry weight. 50 ml of broth were filtered, mycelium washed with water and dried at 85 °C to constant weight.

Alkaloids: Culture filtrate suitably diluted (2 ml) was mixed with VAN URK reagent (4 ml) being prepared as described by AGURELL (1966) and the blue color determined spectrophotometrically with reference to the standard solution of ergotamine tartrate. Mycelial alkaloids were extracted from washed and homogenized mycelium with a mixture of acetone and 4% tartatric acid (1:1). The collected extract was properly diluted with water and used for alkaloid determination as described above. The same procedure as for mycelium was also used for the alkaloid determination in a single colony.

The composition of alkaloids in filtrate and mycelium was revealed by TLC and densitometric scanning (PROŠEK *et al.* 1977).

The method developed is based on the supposition that some very active *Claviceps* strains are capable of forming alkaloids during their growth on solid medium already. The presence of alkaloids was confirmed by extracting single colonies and by colorimetric determination of alkaloids in the extract.

For developing an agar plate method several indicators had been used for the detection of alkaloids in the colonies, such as bromcresol purple, bromcresol blue, gentiana violet, phenol red, fluorescein and 2′, 7′-dichlorofluorescein. With the last two indicators added to the solid medium the alkaloids are detectable in the UV light of 254 nm as dark spots on the fluorescent background. Better response was observed with fluorescein giving blue fluorescence on the yellow fluorescent background.

Table 1

Correlation between alkaloid content and fluorescence intensity in *Claviceps purpurea* colonies growing on solid medium with the fluorescent indicators added

Age of colony days	UV fluorescence intensity 254 nm	Alkaloids[1]) µg/colony
8	+	35
12	++	90
16	+++	180
21	+++	320

[1]) Average of 10 colonies tested

Table 2

Effect of fluorescent indicators on the activity of *Claviceps* colonies

Colony No.	Solid medium	Alkaloid production in the submerged culture (mg/l)			Biomass (g/l)
		Filtrate	Mycelium	Total	
751	SP	399	1182	1581	34.20
752	SP	357	1043	1400	32.10
753	SP	394	1035	1429	32.77
754	SP + dichlorofluorescein	356	945	1301	31.20
755	SP + dichlorofluorescein	378	1139	1517	34.62
756	SP + dichlorofluorescein	432	1125	1557	36.25
757	SP + fluorescein	399	1084	1483	38.42
758	SP + fluorescein	381	1078	1459	37.70
759	SP + fluorescein	378	1026	1404	35.95

SP = sucrose-peptone agar

Table 3

Effect of fluorescent indicators on the composition of the alkaloids formed in submerged cultures

Colony No.	Solid medium	Submerged Culture	LA + EMT %	ETM %	ESN %	d-ETM %	d-ESN %	ECT %	d-ECT %	ETM Σ%	ESN Σ%	ECT Σ%
751	SP	filtrate	6.3	45.5	7.7	18.3	5.3	13.0	4.0	63.8	13.0	17.0
		mycelium	2.4	52.8	2.9	17.9	2.0	13.4	8.6	70.7	4.9	22.0
752	SP	filtrate	7.0	48.0	5.6	17.8	4.4	13.4	3.8	65.8	10.0	17.2
		mycelium	1.6	52.6	1.7	21.8	0.6	14.3	7.4	74.4	2.3	21.7
753	SP	filtrate	7.6	48.9	6.8	15.9	3.8	13.1	3.9	64.8	10.6	17.0
		mycelium										
754	SP + dichloro-	filtrate	7.4	45.6	7.0	20.9	4.4	11.1	3.6	66.5	11.4	14.7
	fluorescein	mycelium	4.2	53.7	2.6	17.1	0.8	14.6	7.0	70.8	3.4	21.6
755	SP + dichloro-	filtrate	6.6	46.1	6.1	18.0	4.1	15.3	3.8	64.1	10.2	19 1
	fluorescein	mycelium	3.0	53.3	2.6	17.2	0.9	15.9	7.0	70.5	3.5	22.9
756	SP + dichloro-	filtrate	5.4	48.4	6.1	17.7	3.4	14.5	4.5	66.1	9.5	18.9
	fluorescein	mycelium	6.0	44.3	8.7	19.4	4.1	13.9	3.7	63.7	12.8	17.6
757	SP + fluorescein	filtrate	6.9	49.0	7.3	16.6	3.7	13.3	3.3	65.6	11.0	16.6
		mycelium	3.5	54.9	4.4	16.7	1.6	13.6	5.2	71.6	6.0	18.8
758	SP + fluorescein	filtrate	6.7	47.9	8.9	17.1	3.7	12.5	3.1	65.0	12.6	15.6
		mycelium	2.9	56.1	3.4	17.0	1.5	13.2	5.8	73.1	4.9	19.0
759	SP + fluorescein	filtrate	8.3	51.1	7.6	16.3	3.0	11.8	2.0	67.4	10.6	13.8
		mycelium	2.5	50.3	4.9	18.7	1.0	15.6	6.0	69.0	5.9	21.6

SP = sucrose-peptone agar
LA = Lysergic acid
EMT = Ergometrine
ETM = Ergotamine
ESN = Ergosine
ECT = Ergocryptine
d-ETM = Ergotaminine
d-ESN = Ergosinine
d-ECT = Ergocryptinine

When following the growth of colonies on agar plates the first noticable fluorescence appeared after 8 days of cultivation and became more intense with aging of colonies. After extracting the colonies and quantitative estimation of ergot alkaloids it was found that stronger fluorescence corresponded to higher alkaloid yield (Table 1). Selection on the above fluorescence intensity is reliable only when colonies are not pigmented, of course, otherwise all chromophore groups will absorb in the UV light.

By comparing the colonies of the same origin, growing on the standard agar medium and agars with fluorescent indicators added, it was concluded that fluorescein as well as 2′,7′-dichlorofluorescein had no influence on the alkaloid production in submerged cultures, neither on quantity (Table 2) nor on the composition of alkaloids formed (Table 3). It means that such low concentration of fluorescein (0.075 mM) or 2′,7′-dichlorofluorescein (0.067 mM) did not inhibit the growth neither the productivity of the fungus *Claviceps* as it was reported by some investigators (PAŽOUTOVA *et al.* 1980), who, however, added fluorescein into submerged culture medium in much larger concentration.

The method described was succcessfully applied for testing colonies of the non sporulating strain of *Claviceps purpurea*. Presumably this technique can also be applied for all *Claviceps* strains capable of forming alkaloids during their growth on solid medium.

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