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The effect of citric acid concentration and pH on the submerged production of lysergic acid derivatives

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Summary. Succinic acid can just as well be replaced by citric acid in submerged fermentation of lysergic acid derivatives by a strain of *Claviceps paspali*. The highest alkaloid yields were obtained with a 1% citric acid concentration in the medium at a constant pH of 5.2. When the optimal pH was not maintained, growth was inhibited and all aspects of metabolic activity of the fungus were depressed.

Introduction

Various strains of the fungus Claviceps exhibit a characteristic carbon source requirement in submerged culture for both growth and alkaloid production. In addition to carbohydrates such as sucrose, mannitol or sorbitol, an organic acid component that is a common intermediate of the citric acid cycle is required for optimal growth and alkaloid synthesis (Arcamone et al. 1970; Pažoutova and Rehaček 1981).

For *C. paspali* strains producing simple lysergic acid derivatives (LADs), succinate is mostly used to supplement the culture media (Arcamone et al. 1961; Brar et al. 1968). Citric acid, which is favourable for *C. purpurea* strains, has been found to inhibit the growth of *C. paspali* and its alkaloid production (Arcamone et al. 1961; Amici et al. 1967; Glund et al. 1979; Kybal et al. 1981). It appears that the influence of organic acid is species-specific (Glund et al. 1979).

The aim of our investigation was, therefore, to find out if succinic acid can be replaced in the production medium by citric acid, which is favourable from the economic point of view. Since the metabolic acitivity with respect to growth and alkaloid synthesis also depends on the citric acid concentration (Glund et al. 1979), in this paper we also discuss the optimal concentration of citric acid for the submerged production of LADs.

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Materials and methods

Microorganism and culture conditions. Claviceps paspali (Stevens and Hall) selected strain ATCC 13892 was used. This strain forms no conidia in submerged cultures and is capable of producing a maximum of 2,000 mg/l of simple LADs under the culture conditions described.

Cultures were kept on potato-glucose agar plates at 24° C and 18-day-old cultures were used for inoculating seed flasks.

Fermentation experiments were carried out in two stages: seed-stage fermentation for 6 days and production-stage fermentation for 14 days. Cotton-wool-plugged 500-ml Erlenmeyer flasks containing 100 ml of culture medium were used for shake cultures. The course of fermentation was followed in a glass fermentor (CHEMAP) with a total capacity of 14 l, an air flow of 1 l/l/min and a rotation velocity of 250 rpm. All fermentations were carried out at 24° C.

Culture media. The seed-stage medium was composed of 40 g/l mannitol, 10 g/l glucose, 10 g/l succinic acid, 3 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7\ H_2O$ and 2 g/l chicken peameal in tap water; the pH of 5.2 was adjusted with ammonia before sterilization.

The production-stage medium contained: 50 g/l mannitol, 30 g/l succinic acid, 1 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7\ H_2O$ and 20 g/l NaCl in tap water. During the experiments, succinic acid was replaced by various concentrations of citric acid; the pH of 5.2 was adjusted with ammonia. Both media were modified according to the method of Arcamone et al. (1961).

Analytical methods

1. The concentration of the *citric acid* was determined according to the method of Sočič and Gaberc-Porekar (1981).
2. The amount of *mannitol* in the culture filtrate was estimated by the polarimetric method of Richtmyer and Hudson (1951), as modified by Arcamone et al. (1961).
3. *Inorganic phosphate* was measured by the method of Berenblum and Chain (1938).
4. *Nitrogen* (NH_4^+) was estimated by the method described by Banks et al. (1974).

5. *Mycelial dry weight:* a sample of culture broth (50 ml) was filtered through filter paper in a Büchner funnel, washed twice with water and dried at 85° C to constant weight.
6. *Alkaloids:* culture filtrate suitable diluted (2 ml) was mixed with van Urk reagent (4 ml), which was prepared as described by Agurell (1966), and the blue colour determined spectrophotometrically with reference to a standard solution of ergometrine base.

Results and disucssion

As shown in Table 1, succinic acid can just as well be replaced by citric acid in the production medium used for a strain of *C. paspali*. Further fermentations conducted with various citric acid concentrations, also in shake cultures, demonstrated that the highest alkaloid titres were yielded at a 1% citric acid concentration (Table 2). With higher or lower citric acid concentrations in the medium, differences were noted in both growth and alkaloid production. Although the specific activity of the strain increased with increasing citric acid concentration, the highest absolute alkaloid yield was achieved with a 1% concentration due to higher biomass formation. From these results it is evident that citric acid plays an important role in the metabolic regulation of the biosynthesis of LAD, being the key regulation element in primary metabolism (Kybal et al. 1981).

When following the course of fermentation in a medium supplemented with a 1% citric acid concen-

Table 1. The influence of the organic acid on the growth of *Claviceps paspali* and the production of lysergic acid derivatives[a]

3% succinic acid			3% citric acid		
Biomass (g/l)	Alkaloid (mg/l)	Sp.act.[b] (mg/g)	Biomass (g/l)	Alkaloid (mg/l)	Sp.act. (mg/g)
6.04	769	127.3	6.30	836	132.7
6.38	850	133.2	5.88	833	141.7
8.92	1,053	118.1	7.57	1,122	148.2
7.44	1,025	137.7	8.40	1,108	131.9

[a] Average of repeated fermentations
[b] Specific activity (alkaloid/dry weight of biomass)

Table 2. Effect of various concentrations of citric acid on the growth of *C. paspali* and the production of lysergic acid derivatives

Citric acid concentration								
0.5%			1%			2%		
Biomass (g/l)	Alkaloid (mg/l)	Sp.act.[a] (mg/g)	Biomass (g/l)	Alkaloid (mg/l)	Sp.act. (mg/g)	Biomass (g/l)	Alkaloid (mg/l)	Sp.act. (mg/g)
13.8	756	54.8	13.1	1,312	100.2	9.7	1,297	133.7
13.1	730	55.7	12.6	1,378	109.4	9.1	1,267	139.2
12.1	736	60.8	11.5	1,286	111.8	9.2	1,283	139.5
13.8	718	52.0	12.6	1,269	100.7	9.5	1,228	129.3

[a] Specific activity (alkaloid/dry weight of biomass)

Table 3. Course of *C. paspali* fermentation in a medium containing a 1% citric acid concentration, without pH regulation

Incubation time (days)	pH	Biomass (g/l)	Alkaloid (mg/l)	Sp.act.[a] (mg/g)	Mannitol (g/l)	Citric acid (g/l)	KH_2PO_4 (g/l)	NH_4^+ (g/l)
0	5.2	1.6	–	–	49	9.5	0.993	1.538
1	5.15	–	–	–	–	8.7	0.995	1.484
3	4.8	5.1	82	16.1	44	8.7	0.148	1.185
5	4.6	8.0	219	27.4	34	8.5	0.132	0.862
6	4.3	8.8	335	38.1	–	–	–	–
7	4.1	8.4	450	53.6	28	8.0	0.090	0.765
8	4.1	8.2	579	70.6	–	–	–	–
9	4.0	7.9	631	79.9	14	7.5	0.000	0.614
10	3.9	7.6	751	98.8	–	–	–	–
11	3.8	7.5	896	119.5	13	7.5	–	0.562
12	3.8	7.5	903	120.4	–	–	–	–
13	3.8	7.4	928	125.4	–	–	–	–
14	3.8	7.4	956	129.2	10	7.5	–	0.391

[a] Specific activity (alkaloid/dry weight of biomass)

tration, a strong acidification was noted if the pH was not regulated. The initial pH of 5.2 dropped markedly, inhibiting both culture growth and LAD production. As shown in Table 3, the overall metabolic activity of the fungus was repressed at the low pH. The inhibiting effects of pH, as discussed earlier by Butlin (1967), are probably not caused by excessive concentrations of hydrogen ions but are due to undissociated acid, which can enter the cell more easily than hydrogen ions and alters the internal pH of the cell. A consequence of this effect is the decrease in biomass, which is probably not connected as much with the lysis of the cells as with the fact that secondary metabolites of the fungus *C. paspali* are excreted into the filtrate.

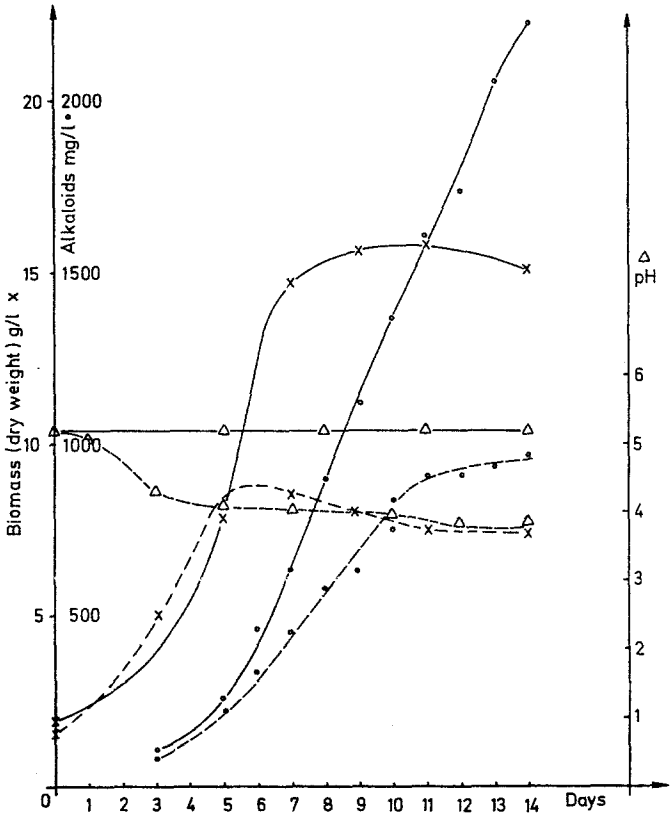

Fig. 1. The influence of pH on the course of fermentation of lysergic acid derivatives in a medium containing a 1% citric acid concentration. ——— Regulated pH; - - - - - Non-regulated pH

In fermentations where the initial pH of 5.2 was adjusted with ammonia and regulated with NaOH, the medium containing a 1% citric acid concentration proved to be optimal for the growth of our strain and its alkaloid production (Fig. 1). The biomass was twice that obtained in fermentations without pH regulation and the alkaloid yields increased greatly. Since there is no significant difference in the specific activities of both fermentations, the main effect of pH regulation could be ascribed to the maintaining of the growth of the fungus.

The physiological effect of pH regulation could also be deduced from other fungal metabolic activities, as shown in Table 4.

The uptake of nutrients from culture media was intensified at a constant pH of 5.2, although citric acid as an additional carbon source was utilized to only about 18% of the initial concentration. This observation is in contrast to the results obtained with other *Claviceps* strains, as with an ergotamine producer for instance, where the consumption of both carbon sources proceeds simultaneously during the course of fermentation and is very high (Amici et al. 1967; Arcamone et al. 1970; Glund et al. 1979). On the other hand, some strains have been described which lost their ability to utilized citric acid, but in this case there was no production of LAD (Amici et al. 1967). In our case high alkaloid yields were obtained when a constant pH was maintained, in spite of poor utilization of citric acid. It seems that citric

Table 4. Metabolic activities of *C. paspali* in a medium containing a 1% citric acid concentration at a constant pH of 5.2

Incubation time (days)	Biomass (g/l)	Alkaloid (mg/l)	Sp.act.[a] (mg/g)	Mannitol (g/l)	Citric acid (g/l)	KH_2PO_4 (g/l)	NH_4^+ (g/l)
0	1.8	–	–	49	9.5	0.996	1.475
1	–	–	–	–	8.6	0.977	1.261
3	5.1	101	19.8	41	8.5	–	1.082
5	7.8	262	35.6	31	8.0	0.099	0.634
7	14.7	628	42.7	21	8.2	0.000	0.522
8	15.4	899	58.4	–	–	–	–
9	15.6	1,122	71.1	11	8.0	–	0.315
10	15.8	1,366	86.5	–	–	–	–
11	15.8	1,612	102.0	5	7.6	–	0.242
12	15.4	1,735	112.7	–	–	–	–
13	15.2	2,062	135.7	–	–	–	–
14	15.0	2,227	148.5	–	7.6	–	0.162

[a] Specific activity (alkaloid/dry weight of biomass)

acid in the form of ammonium salt serves mainly as a source of ammonium nitrogen.

From the results obtained we can conclude, therefore, that the physiology of the *C. paspali* strain used is specific and different from other strains of the genus *Claviceps*.

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