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The negligible role of carbon dioxide and ethylene in ajmalicine production by *Catharanthus roseus* cell suspensions

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Summary. Removal of gaseous metabolites in an aerated fermenter affects ajmalicine production by *Catharanthus roseus* negatively. Therefore, the role of CO_2 and ethylene in ajmalicine production by *C. roseus* was investigated in 3 l fermenters (working volume 1.8 l) with recirculation of a large part of the exhaust air. Removal of CO_2 , ethylene or both from the recirculation stream did not have an effect on ajmalicine production. Inhibition of ethylene biosynthesis in shake flasks with Co^{2+}, Ni^{2+} or aminooxyacetic acid did not affect ajmalicine production. However, the removal of CO_2 did enhance the amount of extracellular ajmalicine.

Key words: Ajmalicine - Carbon dioxide - Ethylene - Bioreactor - *Catharanthus roseus*

Introduction

An important parameter in plant cell fermentations is oxygen supply. Therefore, large scale fermenters are ventilated with large air volumes. Biomass production on a large scale is often comparable with production in a shake flask. However, yields of secondary metabolites are often lower than in a shake flask. Gaseous metabolites can accumulate in a non-sparged shake flask and are stripped in an air-sparged fermenter. In a previous study it was demonstrated that differences in gas exchange were the cause of a lower ajmalicine production by *Catharanthus roseus* cell suspension cultures (Schlatmann et al. 1993). In that study the ajmalicine production in a fermenter could be restored by mimicking the gas exchange of a shake flask in a fermenter, by recirculating a large part of the exhaust air.

In the present study the role of CO_2 and ethylene in ajmalicine production by *C. roseus* was investigated. CO_2 and ethylene are the most often mentioned gasses produced by plant cells in suspension culture (Payne et al. 1991). Some recent studies have emphasized the importance of CO_2 and ethylene in secondary metabolism (Scragg et al. 1987; Cho et al. 1988; Kim et al. 1991; Kobayashi et al. 1991). However, most studies were carried out with the addition of CO_2 or ethylene (precursors), which may be different from the natural control of these gasses by the plant cells, with respect to timing and concentration.

The air recirculation fermenter offers a good experimental set-up to study the role of CO_2 or ethylene. By selectively removing one gaseous metabolite from the recirculation stream, only one factor is changed, and all other produced gaseous compounds and their possible regulatory mechanisms are still active. When an important gaseous metabolite is removed, a low ajmalicine production is expected, comparable to the production by a standard aerated fermenter culture.

Materials and Methods

Cell Material. The cell suspension culture of *Catharanthus roseus* (L.) G. Don. was obtained from the Department of Plant Molecular Biology, Leiden University. The culture was initiated from seeds, and grown in suspension since 1983. It was subcultured every 14 days by adding 35 ml of suspension to 165 ml of fresh LS medium (Linsmaier and Skoog 1965) supplemented with 2.0 mg/l naphthaleneacetic acid, 0.2 mg/l kinetin and 30 g/l glucose. The medium was adjusted to pH 5.8 before sterilization (20 minutes 121°C). The culture was grown in 1000 ml Erlenmeyer flasks closed with silicon foam stoppers T32 (Shin Etsu, Tokyo, Japan), on a gyratory shaker (100 rpm), at 25°C in the dark.

For all experiments a 14-day-old cell suspension was mixed with induction medium at a 1:5 ratio. The (growth limiting) induction medium contained 80 g/l glucose in LS medium without nitrogen and phosphate source. The medium was adjusted to pH 5.8 before sterilization (20 minutes 121°C).

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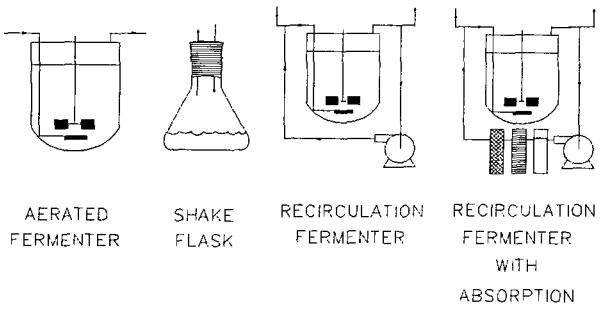

Fig.1. The bioreactors used for the investigation of the role of CO_2 and ethylene in ajmalicine production by *Catharanthus roseus*.

Bioreactors. The bioreactor configurations used in the experiments are shown in Fig. 1. In all bioreactor experiments a 3-l turbine-stirred fermenter was used (Applikon, Schiedam, The Netherlands). Aeration rate of the standard aerated fermenter and the recirculation rate of the air recirculation fermenter were 36 l h^{-1}. Process operation of the standard aerated fermenter and recirculation fermenter (working volume 1.8 l) as well as the 250 ml shake flasks (with 60 ml suspension culture) have been described before by Schlatmann et al. (1993). The experiments lasted for 28 days. For the removal of gaseous metabolites from the recirculation stream, the same set-up has been used as with the recirculation fermenter. In the recirculation stream two filters were positioned, so that the adsorbents could be (re)placed aseptically. Water was removed with a silica column. Gas removal experiments and standard aerated fermenters were started together with a reference experiment: a recirculation fermenter and/or shake flasks, which have a similar ajmalicine production (Schlatmann et al. 1993). As a control, one of the standard aerated fermenter runs was performed with highly purified air. All other bioreactor experiments were performed with pressurized air.

The role of Ethylene. Ethylene was removed by leading the recirculation stream through a column filled with Purafil chemisorbant (Purafil inc., Norcross, USA). The removal of ethylene with Purafil is based on the oxidation with permanganate. Furthermore, as a control for ethylene removal experiments, the effect of inhibition of ethylene biosynthesis was investigated in shake flasks with aminooxyacetic acid (0-40 μM), Ni^{2+} (0, 200, and 400 μM $Ni(SO_4)_2$) and Co^{2+} (0, 100, and 200 μM $CoCl_2.H_2O$). The inhibitors were added to the induction medium before sterilization. The cultures were harvested after 21 days.

The Role of CO_2 . CO_2 was removed selectively by sparging the recirculation stream through 500 ml 2 M KOH. This KOH solution was replaced weekly. The removal of CO_2 was monitored by measuring the exhaust gas continuously with an infrared CO_2 analyzer (Beckman Instruments, Fullerton, USA). The pH was monitored continuously with a sterilizable pH probe (Ingold, Urdorf, Switzerland). Since it is possible that CO_2 can either promote or inhibit ethylene synthesis (Sisler and Wood 1988), a control experiment using the recirculation fermenter with the removal of both CO_2 and ethylene was performed.

Analytical Procedures. The biomass dry weight and the ajmalicine concentration were determined as described by Schlatmann et al. (1993). The biomass and ajmalicine concentrations in the bioreactor cultures were adjusted for the loss of water, by multiplying the concentration with:

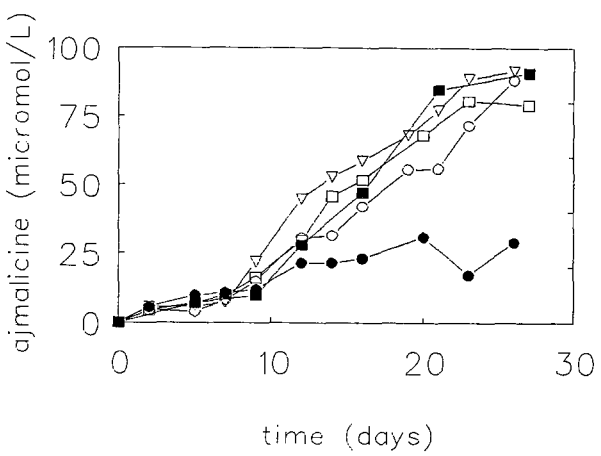

Fig.2. Time course of ajmalicine production by *Catharanthus roseus* in induction medium in: (■) the shake flask, (o) the recirculation fermenter (RF), (▽) RF with removal of ethylene, (□) RF with removal of CO_2 and (•) standard aerated fermenter.

$$\left(1 - \frac{\text{amount of water lost by evaporation}}{\text{starting volume}}\right)$$

Results and Discussion

Fig. 2 shows some typical time courses of ajmalicine production by the different bioreactor cultures as well as the shake flask culture. The volumetric ajmalicine production rate (μmol/l/day) was estimated by linear regression analysis of the time course of ajmalicine production, assuming that the ajmalicine production is constant after induction of the biosynthetic pathway. In Table 1 the results of the experiments are expressed as the ratios of these volumetric production rates in the experiments and their corresponding controls. Using this ratio, possible long-term variation in ajmalicine production by this cell line (Deus-Neumann and Zenk 1984; Morris et al. 1989) is ruled out (the experiments were performed during a one year period).

In the induction medium used for the experiments cell proliferation is not possible. Schlatmann et al. (1994) have shown that an increase in biomass concentration in such an induction medium is a result of the storage of carbohydrates (starch, glucose, fructose, and sucrose). These storage carbohydrates cannot synthesize ajmalicine. Therefore, all ajmalicine biosynthetic capacity was already present in the inoculum, which was the same for all experiments. The average biomass concentration at the start of the experiments was 1.8 ± 0.2 g dry weight/l.

Fig. 2 and Table 1 show that the production by

Table 1. Summary of the results of the gas removal experiments and some control experiments. The ajmalicine production by *Catharanthus roseus* cells in induction medium is expressed as the ratio of the volumetric production rates (calculated from a time course) of the experiment and the control (recirculation fermenter or shake flask, which give the same ajmalicine production (Schlatmann et al. 1993)). The amount of extracellular ajmalicine is expressed as the percentage ajmalicine in the medium of the total amount of ajmalicine.

Experimental Set-up	# Experiments	Ajmalicine Production (%)		Standard Deviation (%)	Extracellular Ajmalicine (%)		Standard Deviation (%)
Standard Aerated Fermenter	4	36	±	12	86	±	19
Recirculation Fermenter with removal of C_2H_4	3	101	±	9	23	±	15
Recirculation Fermenter with removal of CO_2	3	92	±	12	76	±	15
Recirculation Fermenter with removal of C_2H_4 and CO_2	1	99			47		

the shake flask and recirculation fermenter cultures is almost three times higher than by the standard aerated fermenter culture. As a control, one of the four standard aerated fermenter runs was aerated with highly purified air in order to demonstrate that differences between low and high aerated fermenters is not caused by the presence of harmful gasses in the pressurized air system. The production and the extracellular amount of ajmalicine in this run was not significantly different from the other standard aerated fermenters.

The Role of Ethylene

The investigation of the role of ethylene was based on the assumption that *C. roseus* can produce ethylene in suspension culture, although the presence of ethylene in our cultures has not been demonstrated. Numerous studies have shown that ethylene can be synthesized in suspension cultures, with a large variation in the total quantities (Biddington 1992). Removal of ethylene in the recirculation reactor did not have a significant effect on the production of ajmalicine (see Table 1). To test this conclusion, an alternative approach was used: inhibition of ethylene synthesis. Table 2 shows that the inhibition of ethylene biosynthesis does not affect ajmalicine formation. The concentration of the used inhibitors exceeded amply the previously used amounts for successful inhibition: 10-30 μM for Co^{2+} and Ni^{2+} (Yang and Hoffman 1984) and 10-20 μM aminooxyacetic acid (Amrhein and Wenker 1979). The results agree with the results of Piatti et al. (1991) and Songstad et al. (1989), who concluded that ethylene is not needed for secondary metabolism in *Eschscholtzia* and *Papaver somniferum* cell cultures, respectively. Lee and Shuler (1991) found a negative effect of ethylene accumulation on secondary metabolism in *C. roseus*. On the other hand, addition of ethylene or ethylene precursors enhanced secondary metabolism in *Thalictrum rugosum* (Kim et al. 1991; Cho et al. 1988) and *Coffea arabica* (Cho et al. 1988). Apparently, ethylene does not have a general effect on secondary metabolism.

The Role of CO_2

The removal of CO_2 was monitored continuously in the exhaust gas. The removal was very effective, since no CO_2 could be detected (data not shown). Consequently, the dissolved CO_2 concentration was very low, lower than in the standard aerated fermenter. The exhaust

Table 2. The ajmalicine production by *Catharanthus roseus* cells in induction medium in shake flasks, in the presence of several inhibitors of ethylene biosynthesis. The ajmalicine production and biomass dry weight after 21 days are expressed as the percentage of the control shake flasks without the inhibitor (numbers between the parentheses are the standard deviations expressed as the percentage of the total amount. The experiments with aminooxyacetic acid were performed in triplicate, with Ni^{++} and Co^{++} in duplicate).

Ethylene Synthesis Inhibitor	Concentration Inhibitor (μM)	Biomass Dry weight (%)	Ajmalicine Production (%)
aminooxyacetic acid	5	109 (7)	122 (5)
	10	100 (7)	111 (9)
	20	88 (10)	103 (2)
	30	95 (4)	110 (2)
	40	95 (3)	96 (6)
Ni^{++}	200	114 (1)	115 (15)
	400	108 (4)	108 (2)
Co^{++}	100	116 (5)	115 (2)
	200	106 (2)	100 (4)

gas CO_2 concentration of the standard aerated fermenter and the recirculation fermenter were 0.08% and 0.5%, respectively. Surprisingly, the pH of the non-buffered induction medium was not affected by the removal of CO_2. Throughout the experiment, the pH had a constant value of 5.2, which is a normal value for *C. roseus* cultures in this induction medium.

The production of ajmalicine was not significantly affected by the removal of CO_2 (see Fig. 2 and Table 1). A possible interaction between CO_2 and ethylene can be excluded: ajmalicine production was unchanged when both gaseous metabolites were removed. The results of the CO_2 removal experiments are in contrast to the results of other studies using CO_2 enriched air. They reported a beneficial effect of CO_2 on the production of secondary metabolites by *C. roseus* (Scragg et al. 1987), *Thalictrum minus* (Kobayashi et al. 1991) and *T. rugosum* (Kim et al. 1991). Kobayashi et al. (1991) removed CO_2 from a berberine producing shake flask culture of *T. minus*, but, unfortunately, only the effect on culture browning was investigated.

The removal of carbon dioxide has a clear effect on the amount of extracellular ajmalicine, without a visible (microscopic) effect on cell lysis and debris formation. In both the standard aerated fermenter and the recirculation fermenter with removal of CO_2 approx. 80% of the produced ajmalicine was extracellular (low CO_2). In the shake flask, and in the recirculation fermenter with and without ethylene removal, the amount of extracellular ajmalicine was 25%, 23% and 33%, respectively (high CO_2). Reduction of the dissolved CO_2 concentration can affect the intracellular CO_2 concentration, and consequently, the pH of the cell (Bown 1985). Furthermore, control of a constant extracellular pH of 5.2 by the plant cells may have caused an efflux of H^+ from the cells. The combination of the loss of H^+ ions and the reduced intracellular CO_2 concentration can have an effect on the intracellular/vacuolar pH, which increases in this case. Consequently, the ion-trap mechanism to store the ajmalicine, which works at a low vacuolar pH, may have been disturbed (Blom et al. 1991). As a result, ajmalicine is excreted into the medium. Saenz-Carbonell (1993) found a similar release of alkaloids by *C. roseus* hairy roots after a acidification of the medium to pH 3.5, without loss of viability. On the contrary, Cormier et al. (1992) could not release anthocyanins from *Vitis vinifera* without loss of viability by reversing the ion-trap mechanism. As mentioned by the authors, probably the pK of the anthocyanins was too low, i.e. 4-4.3.

In conclusion, the role of both CO_2 and ethylene in ajmalicine biosynthesis by *C. roseus* is very limited. Consequently, the lower ajmalicine production in a well aerated fermenter must have been caused by the removal of an other gaseous metabolite. Plant cells under stress can produce numerous other volatile compounds. For instance, Benson and Withers (1987) have detected methane, ethane, propane, isobutane and pentane amongst several other unidentified compounds in *Daucus carota* cell cultures under cryopreservation stress.

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