Biotechnology Letters Vol 11 No 8 561-566 (1989)
Received as revised July 25th

PRODUCTION OF 4-DECANOLIDE BY SEMICONTINUOUS FERMENTATION OF TYROMYCES SAMBUCEUS

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SUMMARY

Tyromyces sambuceus was cultivated in a stirred tank reactor under semicontinuous conditions for 70 days. Over longer periods of time in submersion, values of 220 and 330 $mg \cdot L^{-1}$ of 4-decanolide could be maintained; after dispersion of the headspace mycelia, a single peak product concentration of 880 $mg \cdot L^{-1}$ was attained. The productivity was subject to large variations, peak values were 18 and 11 $mg \cdot L^{-1} \cdot h^{-1}$. Depending on the culture conditions, specific growth rates of up to 0.05 h^{-1} were reached.

INTRODUCTION

The increasing demand of the food industry for natural aroma substances can no longer be satisfied by the extractive processing of plant materials alone. For this reason, the biotechnological generation of natural aroma substances is attaining increasing significance (Unger, 1980; Schindler, 1982). Among the utilizable organisms, basidiomycetes are known producers of numerous aroma compounds. In particular, basidiomycetes of the order Aphyllophorales, which includes Tyromyces sambuceus, have been demonstrated to be suitable as producers of aroma substances (Drawert et al., 1983; Berger et al., 1986). In addition to several lactones, alcohols, and benzoid substances, Tyromyces sambuceus produces mainly 4-decanolide, a key component of some natural fruit aromas. For economical production of this aroma compound, the parameters

for a long-term cultivation are of particular interest (Jourdain et al., 1985). With the objective of improving the yield, we have therefore investigated the conditions for a semicontinuous fermentation of Tyromyces sambuceus on the scale of a 5 L - fermenter.

MATERIAL AND METHODS

Strain: Tyromyces sambuceus (Lloyd) Imazeki CBS 492.76 N.Hiratsuka CBS Tyromyces s. was precultured in shaking flasks for 14 days forming pellets. As inoculum 60 g Pellets (wet weight) were used (1.5 vvm).

Fermentation: All experiments were performed at 27 °C in a 5L fermenter Biostat V (Braun, Melsungen, FRG) with a working volume of 4L. The fermenter was provided with paddle-stirrers (450-500 1/min) and glass electrodes to control pH and O_2. The air flow was 0.4 vvm. At time intervals of 4-6 days a fixed volume (2 or 4 L) of culture medium plus suspended mycelium was removed and an equal volume of fresh medium was immediately added to the culture. In addition the daily test volume (200 ml) was substituted by 200 ml fresh medium.

Medium: Fermentations were carried out in a modified SNL - medium (Sprecher, 1959) consisting of ($g \cdot L^{-1}$): glucosemonohydrate 20.0, pepton 6.0, KH_2PO_4 1.5, $MgSO_4$ 0.5, thiamin 0.002. In addition 1L medium contained 20 ml castor oil (emulsified with 2 ml Tween 80) and 1 ml of a solution of trace elements (concentrations in $g \cdot L^{-1}$: $FeCl_3 \cdot 6H_2O$ 0.4 $Zn_2SO_4 \cdot 7H_2O$ 0.45, $MgSO_4 \cdot 1H_2O$ 0.152, $CuSO_4 \cdot 5H_2O$ 0.025, Titriplex III 2.0). The pH was adjusted to pH 6.0 with 1N NaOH.

Analysis: Cell concentration was estimated by measuring the dry weight of the mycelium in 100 ml broth. After filtration and washing with H_2O and acetone (removing the remainder of castor oil) the mycelium was dried at 105 °C to constant weight. The specific growth rate was determined using the slope of the dry weight-vs.-time curve (Fencl, 1966).
The 4-decanolide concentration in the fermentation broth was evaluated by means of capillary gas chromatography. After adding an internal standard (1-undecanon) 100 ml broth was steam-distilled. The distillate was extracted with 2 x 50 ml pentane/methylenchloride (2:1). The extracts were desiccated with Na_2SO_4, concentrated to a volume of 0.25 ml using a Vigreux column, and investigated by GLC and GLC-MS. Capillary GLC was carried out using a 23.5 m glass capillary column (0.25 mm i.d.) coated with SE 54 (chemically bound, cross linked) in a Varian 3700 using a linear temperature programme from 40°C to 220°C at 3 °C/min. Hydrogen was used as the carrier gas. Quantities were obtained by the internal standard method using 1-undecanon as the standard component. Mass spectrometry was performed on a Finnigan 1020 (Quadrupole), coupled to a Sigma III gas chromatograph (Perkin-Elmer). The system was equipped with a 30m x 0.32 mm fused silica capillary column, SE 54. Operation conditions were: temperature programme, 5 min isothermal at 65°C, 65 - 220°C at 2°C/min; carrier gas 2 ml/min He; ionization energy 70 eV.

RESULTS AND DISCUSSION

The fermentation was maintained for 70 days from inoculation. During this period the fungus passed through various fermentation phases which are characterized by changing growth rates and productivities (Fig.1 and Fig. 2). Care was taken to ensure that an excess of castor oil was present throughout; ricinoleic acid is supposed to be the precursor for the formation of 4-decanolide (Farbood et al., 1985).

After 60 g of Tyromyces sambuceus (wet weight) had been inoculated into the reactor, the fungus, as expected, passed through a lag phase (1st to 7th day) characterized by low formation of 4-decanolide, slow growth, and an acidification of the medium from pH 6.0 to pH 1.9. At a stirrer speed of 450-500 rpm, regular rod-shaped pellets (2 mm diameter, 5-7mm length) were formed. From the 4th day onwards, discoloration and decreasing product concentration was observed. A removal of medium had become necessary while retaining a sufficient mycelium mass; 2 L of the fermentation broth were pumped out of the reactor and replaced by 2 L of fresh medium. As a result of this procedure, an increase of the pH value to 3.3 was observed; the dry weight of the mycelium was reduced to one half (3.3 $g \cdot L^{-1}$) and the content of 4-decanolide in the medium also decreased in the same ratio from 220 $mg \cdot L^{-1}$ to 110 $mg \cdot L^{-1}$. These direct changes in concentrations are typical for semicontinuous fermentations (Quinlan, 1986). The exchange of the medium proved to be a suitable method for maintaining the viability of the fungus.

The following 17 days of cultivation are divided into three periods characterized by regularly repeating courses of the curves for growth, product formation, and acidification. During the first period (8th to 12th day), a period of adaptation was followed by a period of vigorous product formation whereby a value of 300 $mg \cdot L^{-1}$ of 4-decanolide was reached. In parallel, the dry weight of the mycelium increased to 7.9 $g \cdot L^{-1}$ (average growth rate of approximately 0.92 $g \cdot L^{-1} \cdot d^{-1}$). Since the odour of the air above the medium became more unpleasant from the 11th day onwards, a further 50% of the medium was exchanged on the 12th day. The subsequent second period (13th to 18th day) proceeded analogously to the first period. With an average growth rate of 1.02 $g \cdot L^{-1} \cdot d^{-1}$, dry weight developed up to 9.1 $g \cdot L^{-1}$ and, on the 14th

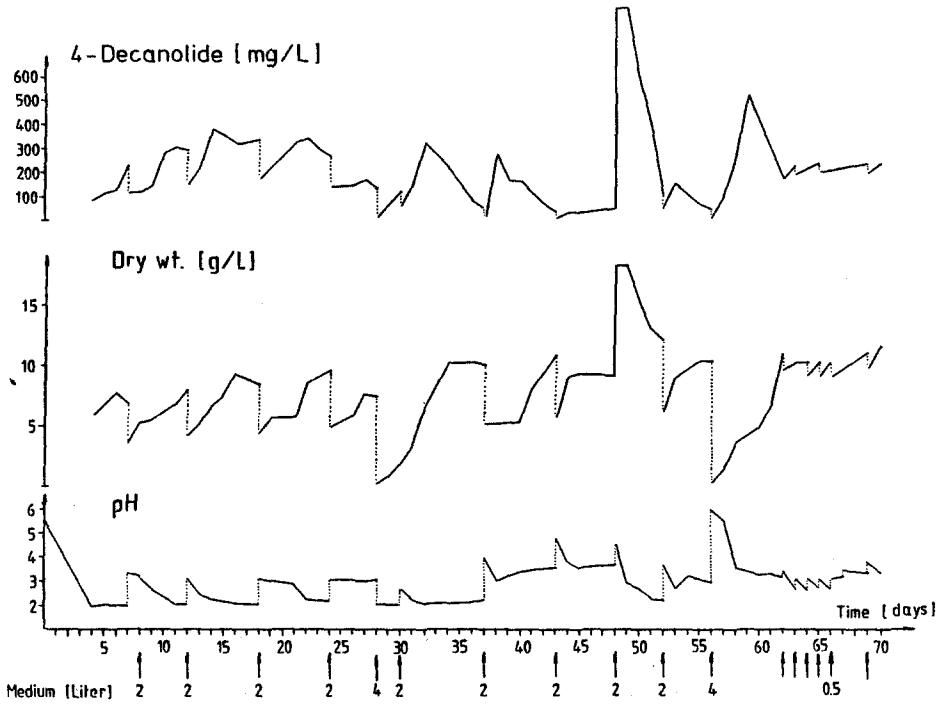

Fig.1 Time course of the concentration of 4-decanolide, dry weight and pH in a semicontinuous culture of Tyromyces sambuceus

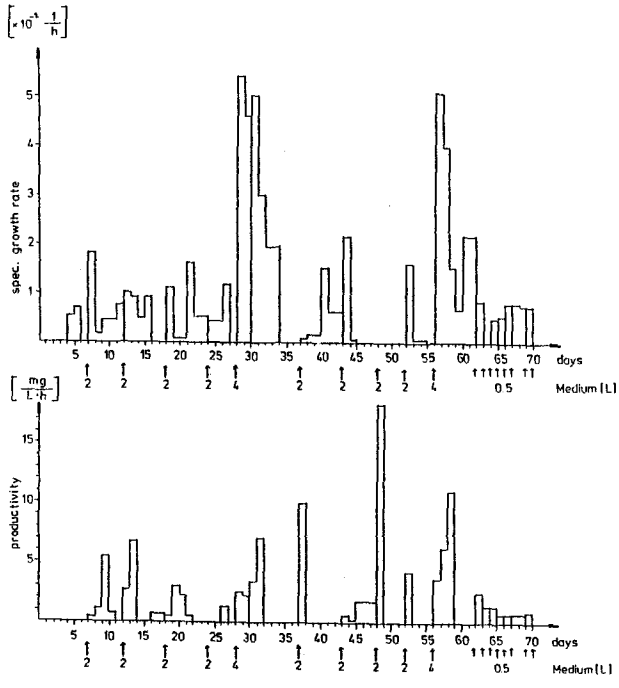

Fig.2 Specific growth rate and productivity

day, a peak value of 372 mg·L^{-1} of 4-decanolide was reached, which subsequently declined. The medium was once more exchanged on the 18th day. The third period (19th to 24th day) again proceeded with similar product formation and pH courses.

As a result of the long duration of the culture, considerable amounts of fungal mycelia accumulated in the headspace of the fermenter. From the 30th day onwards, in particular, the mycelia in the headspace had formed a compact mass and could no longer be washed down into the reactor even by short duration increases of the stirrer speed. Adhesions on the walls and the formation of clumps are rather undesired occurences in the cultivation of basidiomycetes (Germerdonk et al., 1987). The reactor was opened under sterile conditions, the wall adhesions were removed from the headspace, and dispersed in the residual culture broth (48th day). A sample of the broth taken immediately gave a 4-decanolide content of 880 mg · L^{-1}. Obviously a large enrichment of the lactone in the headspace mycelia had occurred; determination of the dry weight gave the high value of 35.6 g·L^{-1}. As a result, the medium had become highly viscous and on the same day, shortly after the sample had been taken, 50% of the medium had to be exchanged.

On the 52nd day, only one-tenth of the peak value of 880 mg·L^{-1} could be measured. A decrease in the mycelium dry weight to 11.3 g·L^{-1} had occurred in parallel to the decrease of the 4-decanolide concentration. This may be attributed to a destruction of the hyphae as a result of the increased shear stress. An increased leakage of fungus cells is to be expected even at stirrer speeds of above 120 min^{-1} (Germerdonk et al., 1987). Thus, a degradation of 4-decanolide by released enzyme systems can be taken into consideration. In this context, the mycelium dry weight of 10-11 g·L^{-1} apparently represents the limiting concentration for the stirrer speed (450-500 min^{-1}). At this mycelium concentration, it is difficult to maintain a sufficient supply of O_2 (König et al., 1981), and this also has a highly deleterious effect on the rate of product formation. The peak productivity value of 18.3 mg·L^{-1}·h^{-1}, which was only achieved after dissolution of the headspace mycelia, is not typical for the long-term submersed cultivation of Tyromyces sambuceus. However, a utilization of this effect must be sought after.

In the continuation of the fermentation (62nd to 70th day), we attempted to subject both growth and lactone production to stable conditions by reducing the time between the medium exchanges. Medium exchanges were performed daily whereby 0.5 L of culture broth was removed and replaced by 0.5 L of fresh medium. A stabilisation of lactone production over several days of the culture was attained. Both the changes of the mycelium dry weight and of the pH curves were highly regular. However, the specific growth rate was only approximately 0.008 h^{-1} and the productivity was 1 $mg \cdot L^{-1} \cdot h^{-1}$. The objective of further experiments will be to stabilize the fermentation at higher productivity levels.

From the work-up of the combined exchanged medium within the 70 days, the recovery of a total 6270 mg of 4-decanolide was possible. A total of 34 L of medium for a total mycelium dry weight of 381 g were required.

Ackowledgement

We are grateful to D. Korz and J.Reischle for technical assistance, and to the BMWi (Federal Minister of Economics) via AiF and FEi for financial support.

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