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Growth and hyoscyamine production of 'hairy root' cultures of *Datura stramonium* in a modified stirred tank reactor

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Summary. The growth and hyoscyamine production of transformed roots of *Datura stramonium* have been examined in a modified 14-l stirred tank reactor in both batch and continuous fermentations on media containing half or full strength Gamborg's B5 salts and at three different temperatures. Under a range of conditions, roots grown on half strength B5 salts with 3% w/v sucrose had a higher dry matter content (up to 8.3% w/w) and a higher hyoscyamine content (up to 0.52 mg·g^{-1} wet weight) than roots grown on full strength B5 salts with the same level of sucrose (up to 4.6% w/w dry matter and up to 0.33 mg hyoscyamine g^{-1} wet weight). Growth at 30° C was initially faster than at either 25° C or 35° C and by day 12, the drained weight of roots in the fermentor at 30° C was about fourfold greater than at 25° C and twice that at 35° C. The ultimate hyoscyamine levels attained (approximately 0.5 mg·g^{-1} wet weight) were similar at both 25° C and 30° C but some 40% lower at 35° C. Final packing densities of 70% w/v were achieved for roots after 37 days growth at 25° C and the highest production rate of 8.2 mg hyoscyamine l^{-1} per day was obtained for roots grown at 30° C. In continuous fermentation at 25° C, the release of hyoscyamine into the culture medium was low (less than 0.5% w/w of the total) but was up to sevenfold higher in fermentors operated at 30° C or 35° C.

Introduction

Considerable interest is now being shown in 'hairy root' cultures for the production of high-value plant secondary products. These cultures offer a high degree of genetic stability, grow rapidly with a profusion of lateral branching and most importantly produce the same spectrum of secondary metabolites in quantities comparable to that of the parent plant (Hamill et al. 1986). So far, few papers have been published regarding the growth of 'hairy roots' in fermentors. The growth of 'hairy root' cultures in fermentors was first reported by Mugnier et al. (1983) for the production of mycorrhizal roots. Rhodes et al. (1986) demonstrated the potential of 'hairy roots' for the production of valuable secondary products following experiments with 'hairy root' cultures of *Nicotiana rustica* in a 1-l fermentor. The roots were first grown as a batch culture and then operated in a continuous mode, during which 76% of the available product (nicotine) was recovered in the effluent at an overall rate of 1.54 mg·l^{-1} per day.

'Hairy roots' of *Calystegia sepium* and *Atropa belladonna* were grown in 2- and 30-l fermentors by Jung and Tepfer (1987). Wilson et al. (1987) reported the growth and production of nicotine in 'hairy roots' of *N. rustica* in 1.5-l column fermentors. These fermentors were operated for up to 65 days in continuous mode. It was found that the continuous phase of operation stimulated product release, leading to a fourfold increase in overall production of nicotine. Taya et al. (1989) operated a 300-ml air-lift column reactor using 'hairy roots' of *Armoracia rusticana* immobilised in polyurethane foam. A root density of 11 g dry weight·l^{-1} was acheived in 31 days.

Preliminary experiments with 'hairy roots' of *Datura stramonium* and *N. rustica* in 14-l stirred tank reactors (Hilton et al. 1988) had shown that damage to the roots could occur through contact with the impeller. This resulted in root disorganisation and callus formation with subsequent loss of productivity. These problems were overcome by using a stainless steel cage to separate the roots from the stirrer and also to provide an immobilisation matrix. In this paper we present data for the kinetics of growth and hyoscyamine production by 'hairy roots' of *D. stramonium* in a 14-l fermentor system developed from those earlier experiments.

Materials and methods

Plant cell cultures and culture media. 'Hairy root' cultures of *D. stramonium*, cell line DS1 (Payne et al. 1987) were maintained by

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regular subculture every 10 days, in 250-ml flasks containing 50 ml of either half ($\frac{1}{2}$ B50) or full strength (B50) hormone-free Gamborg's B5 medium containing 3% (w/v) sucrose (Gamborg et al. 1968). For the fermentor experiments, the roots from four such flasks grown for 8 days at 25° C (between 40–60 g wet weight of roots) were transferred aseptically into the fermentor.

Fermentor modifications. Roots were grown in 14-l glass fermentation vessels, type MF123 (New Brunswick Scientific, Watford, Herts, UK) approximately 200 mm in diameter and 450 mm in height. In order to run these fermentors in a continuous mode and to facilitate drain-and-weigh procedures, several modifications were made to the vessels. These included (a) the addition of two ports (glass B24/29 sockets), one positioned at the 12-l level to provide an overflow weir and another located close to the bottom to allow liquid to be drained from the vessel; (b) alterations to the stainless steel top plate to facilitate the use of autoclavable pO_2 and pH probes; (c) an enlarged stainless steel inoculation port assembly; (d) some modifications to the internal components of the New Brunswick unit. These were carried out as follows: a cylindrical cage, 135 mm diameter × 290 mm high, was constructed from plain weave 0.9 mm diameter stainless steel wire mesh, size no. 4, grade 304 (N. Greening Ltd., Warrington, Lancs, UK) and attached to the inside of the baffles at a distance of 20 mm above the top edges of the impeller blades; a bottom section to the mesh cylinder was also constructed from the same material. The stainless steel mesh cage ensured that the roots did not come into contact with the stirrer blades thus preventing damage, disorganization and subsequent loss of productive capacity. The mesh cage also provided a support matrix for the roots, allowing a more even distribution of the inoculum.

Growth of measurements. The drain port was connected via a length of silicone rubber tubing to a sterile 20-l capacity aspirator. In order to monitor biomass formation during growth, a non-intrusive procedure of draining and weighing was employed. At the times specified, the fermentor vessel was removed from its stand and placed on a top pan electronic balance (0–30 kg in 1 g divisions; Metragram Instruments, Aspley Guise, Bucks, UK) and the running weight recorded. Whilst sparging with air ($2\,l \cdot min^{-1}$), the culture fluid was allowed to drain into the sterile 20-l aspirator and the drained weight subsequently recorded. Increments in biomass were calculated by differences in drained weights. After weighing, the culture fluid was returned to the fermentor vessel by slight overpressure of the aspirator. Growth rates were determined from $\log_e$ plots of the accumulated drained weights (ln drained wt).

Fermentation conditions and analyses. For both batch and continuous fermentations, the vessel was filled with 12 l of either $\frac{1}{2}$ B50 or B50 medium. Following sterilization and inoculation, the fermentors were operated routinely at 25° ± 1° C unless indicated otherwise. The glass vessel was covered with aluminium foil and the roots grown in the dark. The level of dissolved oxygen in the medium was monitored and adjustments made to the airflow rate ($1–2\,l \cdot min^{-1}$) and the stirrer speed (100–200 rpm) to maintain O_2 at values in excess of 80% saturation relative to air. For the continuous feeding experiments, sterile medium was tansferred from a holding aspirator into the vessel at a point close to the impeller using a peristaltic pump (Watson Marlow type 502S, Falmouth, Cornwall, UK). The flow rate was measured by weighing the effluent collected in a sterile receiving flask in a given period. Samples of effluent were taken at intervals and aliquots frozen until required for analysis. At the end of a fermentor run, the root mass was removed and weighed (wet weight). Samples were taken for dry weight determinations. Samples of medium and of roots were also taken for extraction and then analysed for hyoscyamine by HPLC (Payne et al. 1987). Intracellular hyoscyamine concentrations were determined from the mean value obtained from six samples removed from different positions within the fermentor. Glucose and sucrose levels were measured in the culture fluids using enzymic analysis (Bergmeyer and Bernt 1974).

Results and discussion

Batch fermentations

Growth data for 'hairy roots' of *D. stramonium* cell line DS1 grown in $\frac{1}{2}$ B50 and B50 media are presented in Fig. 1 and Table 1. The major increases in drained weight were observed between days 10–18 in $\frac{1}{2}$ B50. and between days 10–26 in B50. The drained weight increased approximately logarithmically with time, doubling every 4.1 days in either $\frac{1}{2}$ B50 or B50. This rapid phase of biomass formation accounted for approximately 70%–75% of the final drained weights. Rates of growth then declined rapidly until no further increases in drained weight were measured. The duration of the growth phase and the resulting terminal drained weights appeared to be dependent upon the ionic strength of the media. At day 40, the final drained weights of the roots grown in $\frac{1}{2}$ B50 and B50 were 1.60 kg and 2.66 kg respectively.

A series of batch fermentation experiments, carried out with cell line DS1 (see Table 1, runs A–D) showed that growth in B50 medium produced a greater amount of biomass, measured on a wet weight basis, than growth in $\frac{1}{2}$ B50 medium, even though the amount of

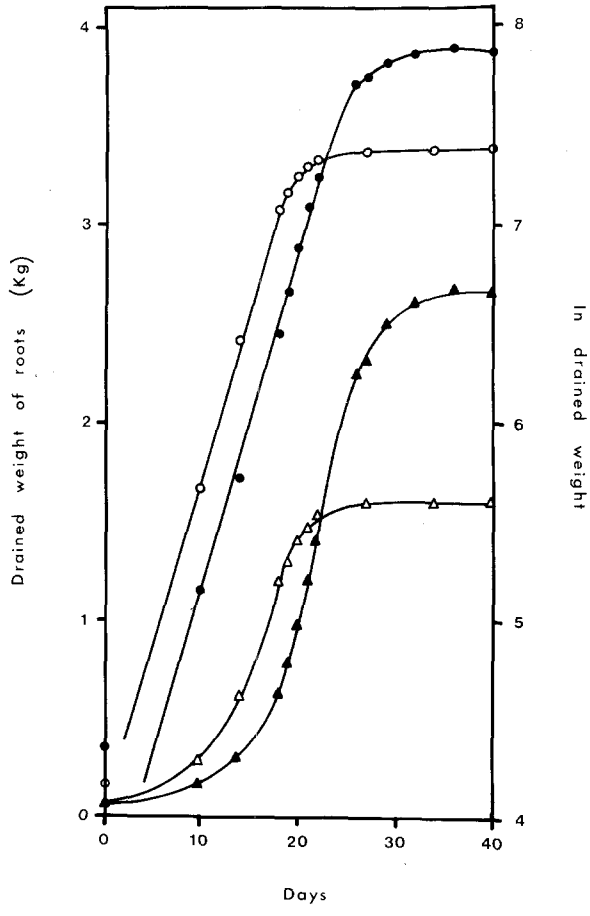

Fig. 1. Batch fermentations at 25° C. Drained weight (△, ▲) and ln drained weight (○, ●) of roots grown in $\frac{1}{2}$ B50 (△, ○) and B50 (▲, ●) media

Table 1. Growth data for 'hairy roots' of *D. stramonium* cell line DS 1

Medium and flow rate ($l \cdot day^{-1}$)	Run	Duration (days)	Drained wt (kg)	Final wet wt (kg)	Final dry wt (kg)	Dry wt/ wet wt (%)
Batch culture						
$\frac{1}{2}$ B50	A	35	1.81	1.45	0.117	8.07
$\frac{1}{2}$ B50	B	40	1.60	1.33	0.087	8.31
B50	C	39	2.03	1.80	0.074	4.11
B50	D	40	2.66	2.05	0.095	4.63
Continuous culture						
$\frac{1}{2}$ B50 (0.6)	E	34	4.24	3.00	0.179	5.97
$\frac{1}{2}$ B50 (1.2)	F	33	5.30	4.30	0.245	5.70
B50 (0.8)	G	37	7.10	5.80	0.248	4.28
$\frac{1}{2}$ B50 (1.2)	$H^{25°}$	24	4.54	3.42	0.195	5.70
$\frac{1}{2}$ B50 (1.2)	$I^{30°}$	22	5.03	3.44	0.177	5.15
$\frac{1}{2}$ B50 (1.2)	$J^{35°}$	23	3.14	2.06	0.103	5.00

available sucrose was the same in both cases. These results are in agreement with Payne et al. (1987) who found that final fresh weight yield was dependent upon the concentration of B5 salts in the medium. However if comparisons between roots grown in $\frac{1}{2}$ B50 with those grown in B50 are made on a dry weight basis, the difference between the two batches of roots is small; indeed the dry weight yield is slightly higher in $\frac{1}{2}$ B50 than in B50. Significant differences were found in the dry weight/wet weight ratios of roots. Those grown in the diluted medium were approximately double the percentage dry weight of roots grown in B50.

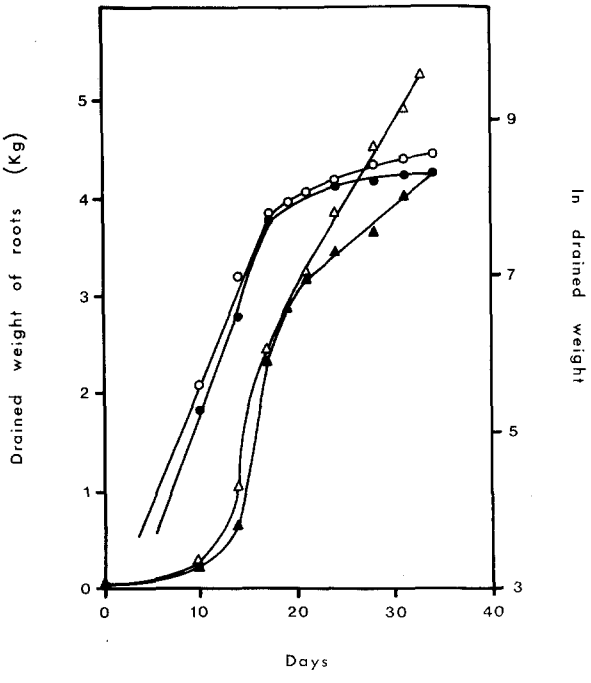

Fig. 2. Continuously fed fermentations at 25° C. Drained weight (▲, △) and ln drained weight (●, ○) of roots grown in $\frac{1}{2}$ B50 at 0.6 $l \cdot day^{-1}$ (▲, ●) and 1.2 $l \cdot day^{-1}$ (△, ○)

Continuous fermentation experiments

As biomass production in the batch fermentations tended to be low, with packing densities of less than 20% (w/v), continuously fed fermentors were set up in an attempt to improve production. The growth of *D. stramonium* DS1 in continuous fermentation at two dilution rates is shown in Fig. 2 and should be compared with the batch runs of Fig. 1. Continuous feeding with $\frac{1}{2}$ B50 was started at day 4, one fermentor at 0.6 $l \cdot day^{-1}$ and the other at 1.2 $l \cdot day^{-1}$. As with the similar batch fermentor runs (Fig. 1) major increases in drained weight were not apparent until after day 10. There was then a rapid increase in drained weight, doubling every 2.1–2.3 days over a period of about 8 days. In both cases, the rates of feeding were sufficient to support very rapid growth but only for a limited period of time. At around day 17, the growth rates began to decline and the roots entered a phase of slower, approximately linear growth. Growth rates during this slower phase were found to be dependent upon the rates of feeding. At 0.6 $l \cdot day^{-1}$ the drained weight increased at a rate of 80 $g \cdot day^{-1}$, and at 1.2 $l \cdot day^{-1}$ the rate was approximately double at 166 $g \cdot day^{-1}$. This gave an average yield of 134 g drained weight of roots per litre of $\frac{1}{2}$ B50.

In order to investigate carbon utilization, effluent samples from the above two fermentor runs were analysed for sucrose and glucose (Fig. 3). Data from a batch fermentation in which liquid samples were removed aseptically from the vessel during the run, have also been included for reference. The level of residual sucrose in the medium was very similar for the batch and the two continuous fermentations even at the highest feeding rate (36 g sucrose $\cdot day^{-1}$). Beyond day 25 less than 0.1% w/v of sucrose was found in the medium in either system suggesting that sucrose utilization remained very high in the fed systems even when growth was slowing down.

Data for the growth of *D. stramonium* from six continuously-fed fermentor runs are shown in Table 1. A

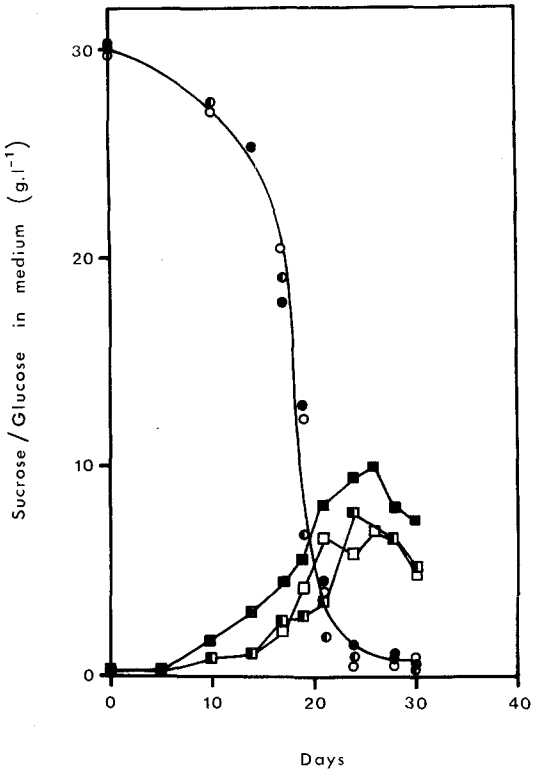

Fig. 3. Sucrose and glucose in the culture medium from batch and continuously fed fermentations in $\frac{1}{2}$ B50 at 25° C. Sucrose: batch (●); 0.6 l·day^{-1} (○); 1.2 l·day^{-1} (◐). Glucose: batch (■); 0.6 l·day^{-1} (□); 1.2 l·days^{-1} (◧)

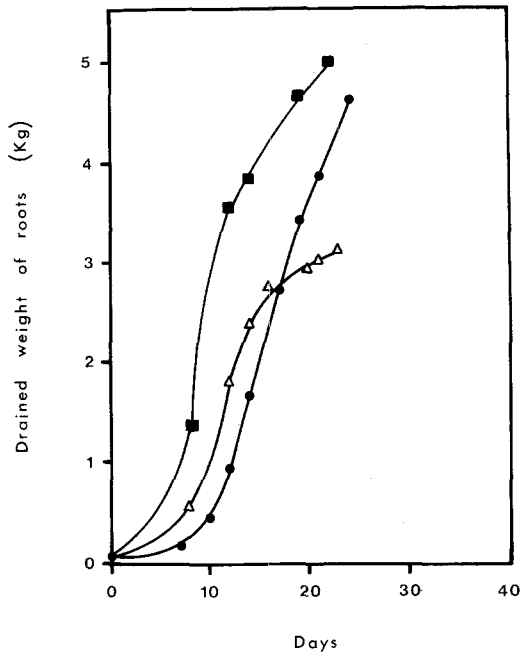

Fig. 5. Drained weight of roots in fermentors continuously fed with $\frac{1}{2}$ B50 at 1.2 l·day^{-1} and operated at 25° C (●); 30° C (■); 35° C (△)

43% increase in wet weight (37% increase in dry weight) was achieved by doubling the feed rate from 0.6 l·day^{-1} to 1.2 l·day^{-1} of $\frac{1}{2}$ B50. Production of biomass was further increased (5.8 kg final wet weight) by feeding with B50 medium (run G, see also Fig. 4). It was possible to increase the packing density of the fermentor to around 70% w/v, an improvement on the low packing densities obtained in the batch systems. Observation of air bubble movement and distribution at these very high densities appeared to indicate a deterioration in the mixing characteristics, thus certain sectors of the fermentor may be exposed to adverse conditions such as low oxygen tension or nutrient levels.

The effect of temperature on root growth was studied in three fermentors containing $\frac{1}{2}$ B50 medium and operated at 25° C, 30° C and 35° C. The roots were allowed to grow as a batch for the first 8 days and were then fed continuously with $\frac{1}{2}$ B50 at a rate of 1.2 l·day^{-1} (see Fig. 5). A high rate of root growth at 30° C was observed soon after inoculation and by day

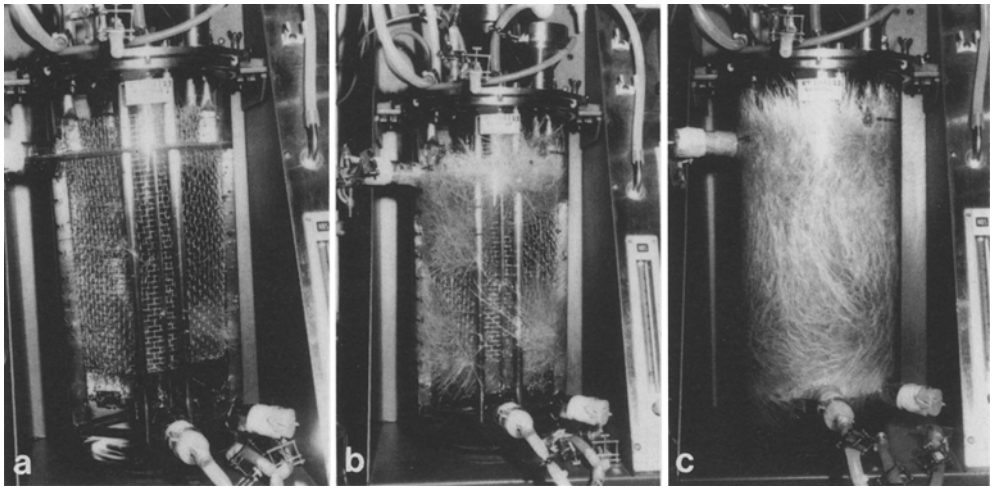

Fig. 4. Growth of *Datura stramonium* DS 1 at 25° C (run G) after 7 days **(a)**; 14 days **(b)**; 28 days **(c)**

12 had already reached a drained weight of 3.5 kg. Such a high rate of growth was not sustainable under these conditions and decreased to an approximately linear rate after day 12. Roots at 35° C grew more slowly than those at 30° C. Again the growth rate declined to an approximately linear rate beyond day 16. Roots grown at 25° C showed the lowest initial rate of growth but since this rate of growth was sustainable for a longer period, the final drained weight was however about 1.4 kg greater than for those roots grown at 35° C.

Differences in growth at the three temperatures were most marked during the first 12–14 days. Figure 6 shows a photograph of the roots at each temperature after 12 days of growth. At 25° C, the roots had immobilized onto the wire mesh and had just started to grow. The roots in the fermentor at 30° C were at a much later stage of growth and had colonized the entire wire mesh. Although the roots grown at 35° C had established themselves throughout the mesh, they had not yet reached the same degree of high density growth found in the fermentor at 30° C. Weight data (Table 1) obtained after harvesting showed similar final wet weights for the roots grown at 25° C and 30° C (approximately 3.42 kg) but only 2.06 kg wet weight from the roots at 35° C.

Hyoscyamine production

Data for hyoscyamine production in *D. stramonium* under the various fermentation regimes is given in Table 2. Batch fermentations produced very similar amounts of total hyoscyamine (0.56–0.67 g). Hyoscyamine was not however detected in any of the samples of culture medium from the batch runs. Amounts of less than 1% w/w of the total have been reported for culture media from small volume flask experiments (Payne et al. 1987). Data from the same flask experiments showed that 32–50 mg of total hyoscyamine were produced per litre of B50 medium compared to values of 47–56 mg of hyoscyamine per litre of B50 in fermentors. These results indicated that scaling up from 0.05 l to 12 l did not lead to any significant loss in hyoscyamine production. In the batch experiments, the total amount of hyoscyamine produced and the rate of production were approximately the same for roots grown on either B50 or $\frac{1}{2}$ B50. The differences in hyoscyamine concentration expressed on either a wet weight or dry weight basis largely reflects the differences in dry weight/wet weight ratios of the roots grown in the two media.

An approximately threefold improvement in both total hyoscyamine accumulation and its rate of production was achieved by operating in the continuous mode. Doubling the flow rate from 0.6 $l \cdot day^{-1}$ to 1.2 $l \cdot day^{-1}$ increased the total amount of hyoscyamine formed and the rate of production by about 50% simply as a consequence of increased biomass formation. However, the fact that roots grown in $\frac{1}{2}$ B50 medium contained 40%–60% more hyoscyamine per unit of wet weight than roots grown in B50 has very important implications in process control. For example, it would be less advantageous to fill a fermentor with B50-grown roots than it would be packing to the same density with roots grown in $\frac{1}{2}$ B50. The total amount of hyoscyamine produced in each case would differ by a factor of about 50%. Such differences in alkaloid concentrations are probably attributable to differences in biomass production in response to varying strengths of media constituents. Nutritional factors may affect such things as the number of lateral branches per unit length or the average cross-sectional dimensions of the roots. The possible variations in root morphology in response to nutritional factors is under investigation and the results will be published later.

The effects of temperature on hyoscyamine production were most marked at the highest temperature tested (35° C). The concentration found in the roots was approximately 60% of that found at 25° C and combined with lower final root yield, the total amount found in the system was about 60% below that at 25° C. At 30° C, the concentration of hyoscyamine was, if anything, slightly enhanced and coupled with the rapid

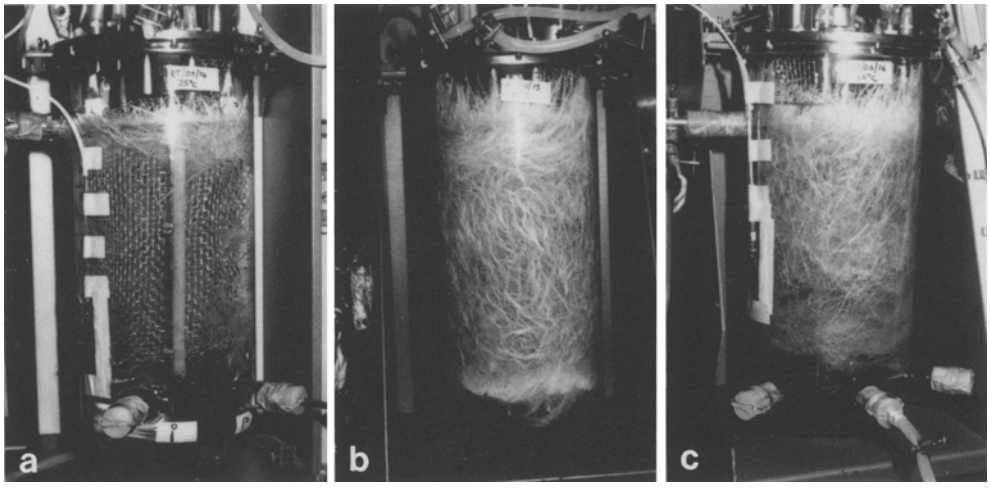

Fig. 6. Growth of *D. stramonium* DS 1 at 12 days in continuously fed fermentors operated at 25° C **(a)**; 30° C **(b)**; 35° C **(c)**

Table 2. Data for hyoscyamine production in *D. stramonium* under various fermentation regimes

Medium and flow rate ($l \cdot day^{-1}$)	Run	Hyoscyamine				
		Total in system (g)	% Total in medium	Concentration in roots		Productivity[a] ($mg \cdot l^{-1} \cdot day^{-1}$)
				(mg/g wet wt)	(mg/g dry wt)	
Batch culture						
$\frac{1}{2}$ B50	A	0.67	nd	0.46	5.7	1.9
$\frac{1}{2}$ B50	B	0.56	nd	0.42	6.4	1.4
B50	C	0.60	nd	0.33	8.1	1.5
B50	D	0.65	nd	0.32	6.9	1.6
Continuous culture						
$\frac{1}{2}$ B50 (0.6)	E	1.56	0.4	0.52	8.7	4.6
$\frac{1}{2}$ B50 (1.2)	F	2.12	1.2	0.49	8.7	6.4
B50 (0.8)	G	1.80	0.6	0.31	7.3	4.6
$\frac{1}{2}$ B50 (1.2)	$H^{25°}$	1.62	0.5	0.47	8.3	6.7
$\frac{1}{2}$ B50 (1.2)	$I^{30°}$	1.81	2.5	0.51	10.2	8.2
$\frac{1}{2}$ B50 (1.2)	$J^{35°}$	0.63	3.6	0.30	6.2	2.8

[a] Amount of hyoscyamine produced per litre of the fermentor working volume per day

growth observed at that temperature, gave the highest productivity so far observed (8.2 mg hyoscyamine/l per day). The amount of hyoscyamine found in the medium as a percentage of the total increased with operating temperature. At 35° C the amount of hyoscyamine found in the medium collected during fermentation was 3.6% w/w of the total. This was over sevenfold higher than the corresponding amount at 25° C (0.5% w/w).

Figure 7 shows the accumulated amounts of hyoscyamine found in the effluents from the three fermentors over a period of 16 days. At 30° C and 35° C the amount of hyoscyamine exported into the medium was 0.8 $mg \cdot day^{-1}$ and at 25° C, the rate was much lower at 0.2 $mg \cdot day^{-1}$. The higher operating temperatures may have increased the permeability of the cells to hyoscyamine allowing increased transport of product into the medium.

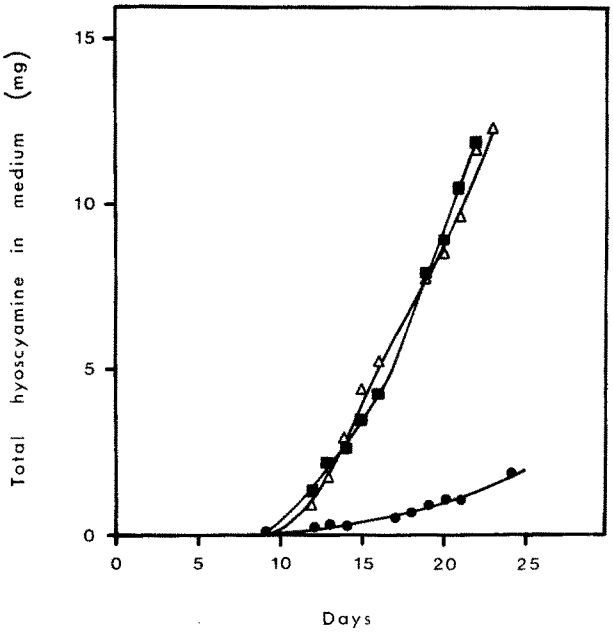

Fig. 7. Hyoscyamine in the effluents collected from fermentors continuously fed with $\frac{1}{2}$ B50 ($1.2\,l \cdot day^{-1}$) and operated at 25° C (●); 30° C (■); 35° C (△)

Further improvements in the levels of intracellular hyoscyamine may be possible through the development of a high-producing cell line by selection or genetic engineering. Operating fermentors with cell lines capable of exporting large amounts of alkaloid into the medium, perhaps at elevated temperatures to enhance release, would offer the best operational system. A continuous flow system would then operate with the original biomass using nutritionally defined media capable of maintaining viability and synthetic capacity within the roots.

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