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Optimisation of fermentation conditions for the production of a novel GABA-benzodiazepine receptor agonist by *Acremonium strictum*

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The production of XR368, a novel GABA-benzodiazepine receptor agonist, has been optimised through manipulation of the medium composition and fermentation conditions leading to an increase in product titre from 1 to 100 mg L^{-1}. Key steps in the optimisation process were the selection of carbon and nitrogen sources, and the optimisation of the C:N ratio and medium concentration. Furthermore, downstream processing has been facilitated through the introduction of a detergent treatment step which results in the release of over 90% of the desired product into the extracellular environment, avoiding the need to process both biomass and liquor fractions.

Keywords: fermentation optimisation; sesquiterpene-polyketide; XR368

Introduction

XR368 (Figure 1) is a mixed sesquiterpene-polyketide metabolite which was discovered during high throughput screening of microbial samples for inhibitors of the binding of benzodiazepines to the GABA benzodiazepine ionophore receptor complex [1,6]. Although related strains in our culture collection have been investigated for production of this compound, XR368 was detected exclusively in cultures of *A. strictum* X06/15/458. In order to conduct structure-activity relationship studies and to evaluate the biological potency and pharmacological properties of compound XR368, large quantities of the compound were needed. Starting with an initial product titre of only 1 mg L^{-1}, which is not uncommon for microbial products, the challenge was to rapidly develop a fermentation process yielding substantially increased product titres at favourable costs. This paper describes the most influential investigations involved.

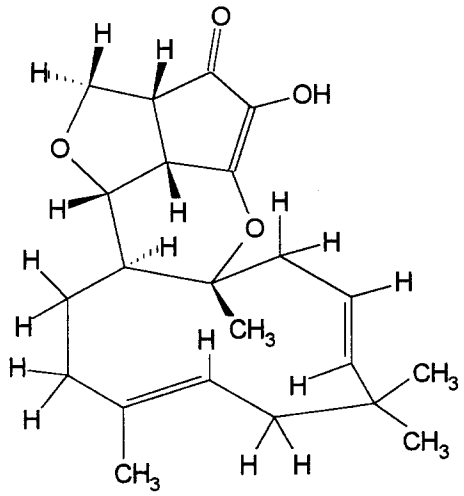

Figure 1 Compound XR368.

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Materials and methods

Producing organism

The fungus has been referred to as *Acremonium strictum* W Gams (Xenova number X06/15/458). The strain was deposited at the International Mycological Institute (Egham, Surrey, UK) under the accession number IMI 354451.

Media

The original seed medium contained (g L^{-1}): glycerol 15, D-glucose 10, malt extract 5, soybean peptone 15, NaCl 3, $CaCO_3$ 1, Junlon PW110 (Honeywill and Stein, Sutton, UK) 1 and 0.1% v/v Tween 80 (Sigma, Poole, UK), adjusted to pH 6.0 with sulphuric acid before sterilisation.

The original production medium contained (g L^{-1}): trehalose 23.75, yeast extract 6.9, 2-[N-morpholino]ethanesulphonic acid (MES) 9.75, carboxymethylcellulose 1 and 0.1% v/v Tween 80 adjusted to pH 6.0 with sulphuric acid before sterilisation. When testing the effect of medium strength 1× medium contained (g L^{-1}): sucrose 23.75, yeast extract 6.9, MES 9.75, carboxymethylcellulose 1, 0.1% (v/v) Tween 80 and 0.1% (v/v) Antifoam A (Sigma, Poole, UK). The 2× medium was 1× medium with the concentrations of sucrose, and yeast extract doubled. For the seed stage experiment, the production medium contained (g L^{-1}): maltose 45, yeast extract 6.9, K_2SO_4 8.7 and 0.1% v/v Tween 80.

The production medium used for the final production process contained (g L^{-1}): high maltose syrup BR20 140 (Ragus Sugars, Slough, UK), yeast extract 13.8, 0.1% (v/v) Tween 80 and 0.1% (v/v) Antifoam A.

Culture conditions

Conidia were obtained from mycelium grown for 10 days on half-strength potato-dextrose agar, using a 10% glycerol/

0.1% Tween 80 solution. After separation of the remaining mycelium by filtration through non-absorbent cotton wool, the conidial suspension was adjusted to a concentration of 10^8 conidia ml^{-1} and stored at −135°C. Seed cultures in test tubes were inoculated at a final concentration of 10^7 conidia ml^{-1} and incubated at 25°C and 240 rpm on a shaking incubator.

Production cultures in test tubes were inoculated using seed cultures grown for 72 h and incubated under the same conditions. Details of fermentation conditions for production cultures in fermenters are indicated in the text.

Sample preparation and analysis of XR368

For product analysis from the biomass, the mycelium from a 10-ml fermentation sample was separated from the liquor by centrifugation at 1915 × *g* for 10 min. A 10-ml volume of methanol was added to the biomass fraction, the suspension was sonicated for 10 min, and then filtered to remove all biomass prior to analysis by HPLC.

Product analysis in the liquor was carried out by adding 0.5 ml of methanol to an equal volume of culture liquor. This mixture was centrifuged at 7406 × *g* to sediment any precipitate prior to analysis by HPLC:

Product analysis from the whole culture was performed by adding 200 μl Tween 80 (2% v/v final concentration) to a 10-ml sample and continuing the incubation for a further 2 h. The sample was then centrifuged as above and an equal volume of methanol was added to a portion of the supernatant phase. The mixture was centrifuged at 7406 × *g* to remove any precipitate prior to analysis.

Analysis was carried out using a Waters HPLC system equipped with a Waters 991 or 996 photodiode array detector and a Waters NovaPak C18 Rad Pak cartridge column. Separation was achieved with a gradient of 50–100% acetonitrile in water over 12 min, giving a typical retention time for XR368 of 7 min.

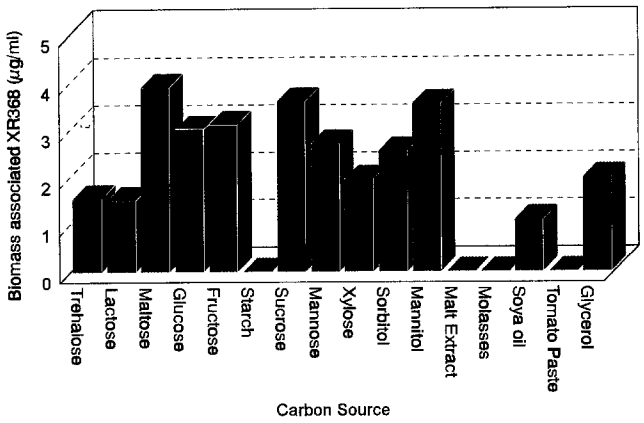

Figure 2 Effect of carbon sources on production of compound XR368. Carbon sources were tested at concentrations calculated to contribute 730 mM carbon to the medium.

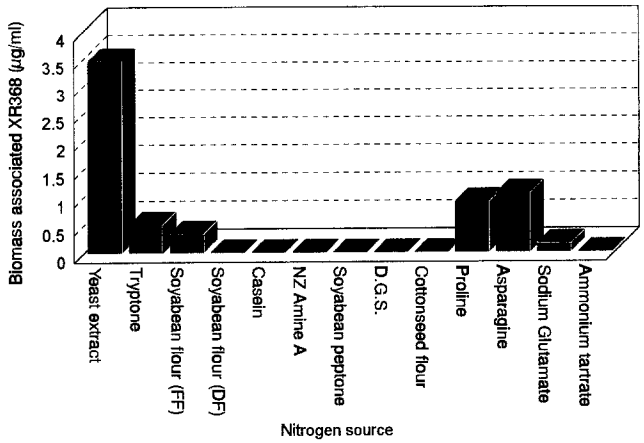

Figure 3 Effect of nitrogen sources on production of compound XR368. Nitrogen sources were tested at concentrations calculated to contribute 50.2 mM nitrogen to the medium. D.G.S. = Distillers grains and solubles.

Results

Initial fermentations

For early fermentations a mixture of mycelium and conidia obtained from an agar culture was used to inoculate seed cultures. The resultant titres of compound XR368 in the production phase were variable and frequently no product was detected. The reproducibility of the fermentations was increased through the adoption of a homogeneous and defined suspension of conidia as inoculum for the seed stage, and titres of 1–2 mg L^{-1} were routinely achieved in fermentations prior to optimisation of the medium composition.

Optimisation of carbon and nitrogen sources

A number of complex and defined carbon and nitrogen sources were compared for their ability to support production of compound XR368 whilst all other media components remained constant (Figures 2 and 3). The carbon and nitrogen sources were added at concentrations calculated so that they would contribute approximately equal amounts of carbon or of nitrogen to the medium. Complex medium components were analysed by Medac Ltd (Brunel University, Uxbridge, UK) for total carbon and nitrogen content and the results are shown in Table 1. Where defined nitrogen sources were used the media were supplemented with inorganic phosphate, vitamins and trace metals. On the basis of product titre and metabolite complexity, as shown

Table 1 Results of analysis of complex medium components by Medac Ltd

Component	Carbon content (% w/w)	Nitrogen content (% w/w)
Malt extract	40.59	0.205
Molasses	31.77	<0.01
Soyabean oil	77.55	<0.1
Tomato paste	13.03	0.735
Yeast extract	38.19	10.20
Tryptone	40.57	11.22
Soyabean flour (full fat)	50.92	6.565
Soyabean flour (defatted)	43.255	8.35
Casein	43.94	12.525
NZ amines	43.9	12.73
Soyabean peptone	40.545	9.215
Distillers grains and solubles	43.035	3.805
Cottonseed flour	44.79	9.23

by chromatographic profiles, yeast extract was chosen as nitrogen source and sucrose and maltose were both investigated further as carbon sources. Although titres on the two sugars were similar, maltose was eventually chosen because fewer related compounds were produced on this carbon source.

At a later stage alternative maltose sources were tested in order to reduce medium costs. Of seven different maltose samples tested, each supported similar levels of XR368 production (data not shown). A food grade product, high maltose syrup BR20, available at a fraction of the cost of purer preparations was chosen for the final production process.

Medium pH

The initial pH of the production medium was varied in order to assess its effect on the production of XR368. The results of this study are shown in Figure 4. As the initial medium pH was 6.0 in the original fermentation conditions, this remained unchanged.

Medium strength

In order to improve the volumetric productivity in the fermenter, we tested the effect of different medium concentrations on both growth and production. This experiment was carried out in two identical 20-L fermenters, each containing 16 L of medium. The air flow rate was 8 L min^{-1} and the stirrer was set at 400 rpm. Each fermenter was inoculated with 640 ml of a 72-h-old culture grown in a 3-L fermenter containing 2 L of the original seed medium at 28°C, airflow rate 1 L min^{-1} and 500 rpm stirrer speed.

Doubling the medium concentration resulted in a doubling of the final biomass in the fermenter (Figure 5) and maintenance of production for an extended period. As a result, the final concentration of compound XR368 in the 2× concentrated medium was increased by a factor of 3 after 7 days of fermentation. Further experiments (data not shown) using higher media concentrations indicated that the 2× concentrated medium was optimal.

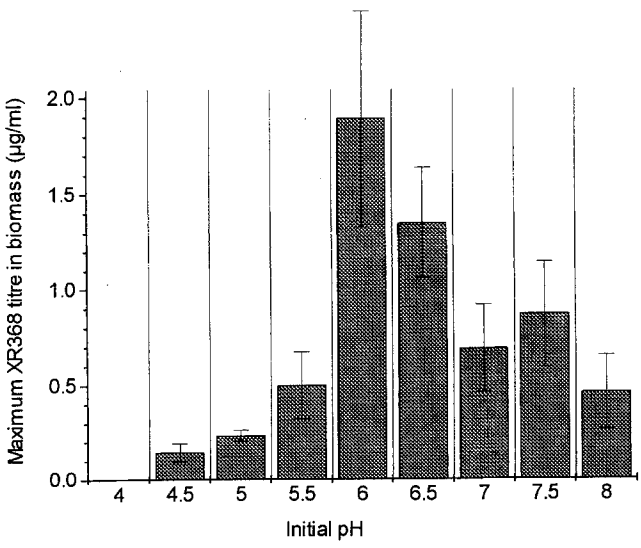

Figure 4 Effect of initial medium pH on titres of compound XR368.

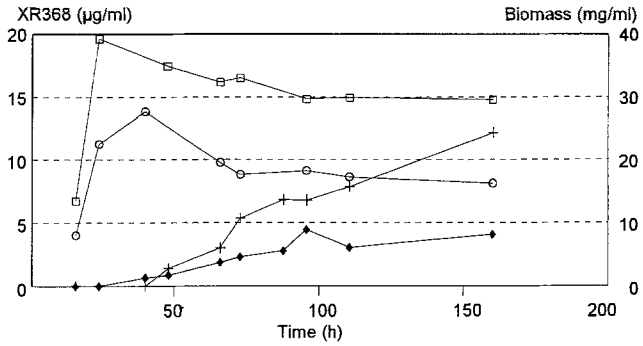

Figure 5 Fermentation profiles on media at different concentrations. Biomass in single (○) and double-strength medium (□). Production in single (◆) and double-strength medium (+) (biomass-associated compound XR368 only).

Optimisation of C:N ratio

The effect of the carbon to nitrogen (C:N) ratio was investigated by varying the amount of maltose in the medium. The amount of yeast extract was kept constant in order to minimise the effect of variation in biomass level on product titre. The ratio in the original medium was 19 : 1. Results for media with C:N ratios between 6.3 and 34.1 :1 and for ratios between 29 and 62 : 1, performed in two separate experiments, are shown in Figure 6, a and b, respectively. These clearly indicate that increased titres of compound XR368 are achieved with higher C:N ratios and the optimum ratio of 40 : 1 was chosen for future studies. Calculated ratios include the total carbon and nitrogen contribution from yeast extract.

Seed stage

The seed stage was optimised with respect to age and inoculum volume for the production phase. The seed was cultivated on the original seed medium and transferred after 2, 3, 4 or 5 days. The inoculation volume for the production culture was 2, 4 or 8%. An incubation time of 3 days and seed volume of 4% of the production volume were found to be best for production (Figure 7). After 3 days the morphology of the seed culture had changed from mycelial growth to a predominantly conidial form.

This morphological switch from hyphal to conidial form had also been observed during the production phase, where it coincided with the onset of production of compound XR368, and we therefore compared the original seed medium with the production medium for cultivation of the seed. Both media supported growth corresponding to a dry cell weight of 6 g L^{-1} and conversion to the conidial form. Nevertheless, the seed grown in the production medium resulted in XR368 titres of less than 2 mg L^{-1} compared to over 45 mg L^{-1} on day 6 (Figure 7) when the original seed medium was used. Compound XR368 was not detected in either seed culture.

Further experiments (data not shown) showed that Junlon PW110 could be omitted from the seed medium without detrimental effect on final product titre.

Medium formulation

A Plackett-Burman [4,5] design experiment was used to assess the importance of various medium components for

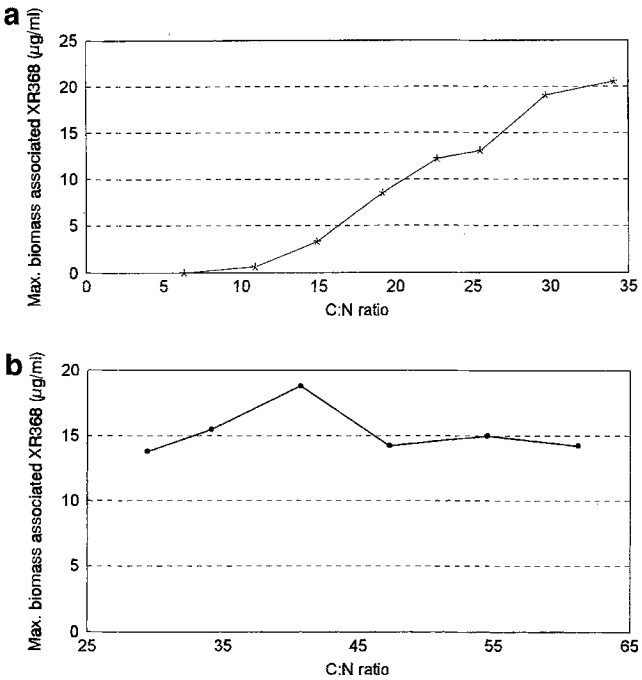

Figure 6 (a) Effect of C:N ratios of between 6.3 and 34.1 on titres of compound XR368 after 7 days incubation. (b) Effect of C:N ratio of between 29.4 and 61.2 on titres of compound XR368 after 7 days incubation.

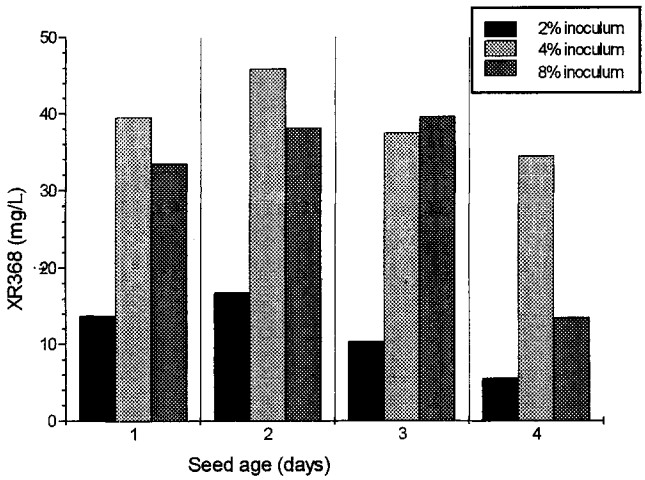

Figure 7 Effect of seed age, and inoculation volume on titres of compound XR368 after a production phase of 6 days. The standard seed medium was used, the seed age was varied between 2 and 5 days and the inoculation volume was varied between 2% and 8%.

the production of compound XR368. This kind of experiment allows the evaluation of a number of medium components using a minimal number of experimental set-ups. Medium components were added to one of two levels in the experimental medium and on analysis of the experiment were given a score according to the magnitude and direction of their effect on product titre. The components, the levels used and their scores are shown in Table 2. Only the negative effect of calcium carbonate with a value of −6.94 was statistically significant, using Student's *t*-test. Other components had no statistically relevant influence on production of compound XR368. Considering costs and ease of

Table 2 Results of Plackett-Burman experiment

Component	+ Value	− Value	Effect
K_2SO_4	8.7 g L^{-1}	0	+2.45
Na_2SO_4	7.1 g L^{-1}	0	+1.705
Tween 80	0.1%	0	+0.238
NaCl	5.84 g L^{-1}	0	−0.36
Carboxymethyl cellulose	1 g L^{-1}	0	−0.674
MES	9.75 g L^{-1}	0	−1.375
$CaCl_2$	20 g L^{-1}	0	−6.94

downstream processing, carboxymethylcellulose and MES were thus omitted from the production medium. The addition of K_2SO_4 to the medium was followed up briefly but produced no significant benefit on scale-up.

Secretion of compound XR368

In early fermentations compound XR368 was detected only in the biomass extract. With the increased titres obtained during the fermentation development programme, significant amounts of the compound were also detected in the liquor, requiring downstream processing procedures to be adapted accordingly. With the aim of releasing the compound into the liquor, the effect of a series of treatments for the permeabilisation of cells [3] was tested. Results are shown in Figure 8 and indicate that more than 90% of the total amount of compound XR368 measured was present in the liquor fraction following addition of nonionic detergents and continued incubation under the same conditions for 2 h. For future processing Tween 80 was the agent of choice over Triton X100, since HPLC profiles of the resultant samples indicated that fewer additional compounds were also being released from the cells.

Production process

The optimised fermentation was carried out in a 75-L fermenter containing 50 L of medium and incubated at 25°C, 350 rpm, airflow rate 25 L min^{-1}. This was inoculated using a 72-h-old 2-L culture grown in a 3-L fermenter on the original seed medium minus Junlon PW110 and incubated

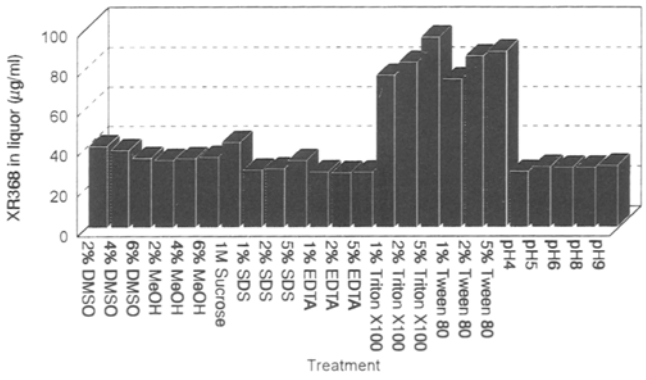

Figure 8 Effect of treatments on release of compound XR368 into the culture liquor. Analysis of the biomass remaining after treatment with 5% Tween 80 showed that the total biomass + liquor titre of XR368 was 103.7 mg L^{-1}. All treatments were carried out on aliquots of the same fermenter sample.

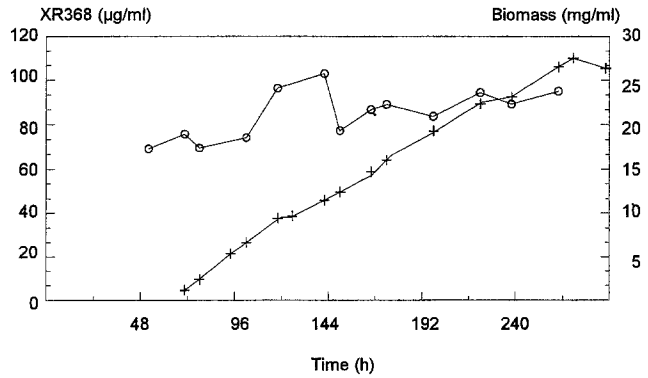

Figure 9 Profile of total compound XR368 (+) and biomass (dry cell weight) (○) in optimised fermentation.

at 28°C, 500 rpm, airflow rate 25 L min^{-1}. The profile of a typical fermentation at this scale is shown in Figure 9.

Discussion

Optimisation of the fermentation procedure for production of compound XR368 was necessitated by the requirement for large amounts of material for evaluation of this lead compound. An integrated approach was applied to the optimisation process with improvements at a test tube scale immediately being translated to larger vessels. Consideration was also given to the simultaneous development of cost-effective downstream processing so that in addition to improvements in product titre, the presence of related metabolites in the fermentation broth were minimised for ease of product isolation. The changes introduced during the course of the process development not only increased titres to over 100 mg L^{-1} but also simplified the production medium and facilitated product isolation. One of the most significant developments in the procedure was treatment of the culture with Tween 80 prior to harvest so that only the liquor fraction required processing.

The nature of the carbon and nitrogen sources in the production medium was one of the first factors investigated, with maltose and yeast extract being chosen from the range of tested nutrients. A large number of the carbon sources tested supported good levels of production of compound XR368, but notable exceptions were the complex sources (molasses, malt extract and tomato paste). It is possible that production of this metabolite was sensitive to nitrogenous compounds, trace metals or vitamins present in these complex ingredients in addition to the carbohydrate components. This was supported by results from the experiments evaluating nitrogen sources where only yeast extract yielded good levels of production.

Investigation of the effect of the medium C:N ratio yielded led to improvements in titres of compound XR368. Higher titres were achieved in media with higher C:N ratios due to an increase in both rate and duration of production. The increased availability of acetate units from the metabolism of the additional carbon source in the higher C:N ratio media is likely to be, at least partly, responsible for these elevated titres. No production of compound XR368 was seen with the lowest C:N ratio tried, possibly indicating the presence of a critical ratio below which production does not take place, as observed for production of milbemycin by *Streptomyces hygroscopicus* [7].

One of the most interesting facets of this work was the unusual morphology displayed by the producing organism. There is no doubt that the morphological switch from mycelial to conidial form seen in both seed and production stages is relevant to the production of compound XR368, but its exact role is yet to be understood. A similar link between morphology and production of a secondary metabolite has been noted for the production of Cephalosporin C by *Acremonium chrysogenum* [2]. The morphological transformations and their relevance to product biosynthesis are better characterised in this system and it is reported that a yeast-like morphological form is most biosynthetically active [2].

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