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A chemically-defined medium for production of compactin by *Penicillium citrinum*

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Abstract

A mutant strain of *Penicillium citrinum* grown in a chemically-defined production medium, yielded 145 mg compactin l^{-1}. The medium also facilitated spectrophotometric analysis of compactin. Addition of KH_2PO_4in the production medium did not increase the compactin production, while addition of a surfactant, Tween 80, increased compactin to 175 mg l^{-1}. Inoculation with 10^7 spores ml^{-1} and initial pH of 6.5–7 were the most suitable for compactin production.

Introduction

Compactin is a specific and potent inhibitor of cholesterol biosynthesis, and also acts as an antifungal agent (Endo *et al.* 1976a, b, Brown *et al.* 1976). Compactin competitively inhibits the regulatory enzyme, 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase. Compactin is also a precursor of pravastatin, which is also an anti-hypercholesterolemic agent. Among the few commercially used microbial strains for the production of compactin are *Penicillium citrinum* (Endo *et al.* 1976a, b, 1985, 1986, Hosobuchi *et al.* 1993a, b, Konya *et al.* 1998), *P. cyclopium* (Hamdy *et al.* 1998) and *Aspergillus terreus* (Manzoni *et al.* 1999).

Attempts have been made to improve the yield of compactin using *P. citrinum* fermented on complex media (Endo *et al.* 1976a, b, 1985, 1986, Hosobuchi *et al.* 1993a, b, Konya *et al.* 1998, Hamdy *et al.* 1998, Manzoni *et al.* 1999). The present investigation highlights the use of defined production medium using simple nitrogen sources to increase the final product concentration and to get a relatively easier spectrophotometric analysis of compactin.

Materials and methods

Organism

Penicillium citrinum NCIM 768 was from NCL, Pune, India. A spore suspension (10^7 ml^{-1}) was mutagenized by UV light (30 W, 254 nm) as per standard procedures. Mutants were screened for their ability to produce compactin and one among the best, designated VR-12 was chosen for the present study on compactin production.

Medium

The culture was grown on potato/glycerol/agar slants and spore suspension was made in 15 g glycerol l^{-1}. The three inoculum media (IM) used in this study were: IM-A medium [glucose, 20 g l^{-1}; glycerol, 30 g l^{-1}; peptone, 8 g l^{-1}; $NaNO_3$, 2 g l^{-1} and $MgSO_4$, 1 g l^{-1} were dissolved in water-soluble extract of soybean meal (40 g soybean meal in cheese cloth was kept in 1000 ml distilled water at 4 °C under stirring condition for 6 d)], IM-B medium (glucose, 20 g l^{-1}; urea, 8.4 g l^{-1}; glycerol, 30 g l^{-1} and $MgSO_4$, 1 g l^{-1}) and IM-C medium (glucose, 20 g l^{-1}; glycerol, 30 g l^{-1}; $(NH_4)_2SO_4$, 18.5 g l^{-1}; NH_4Cl,

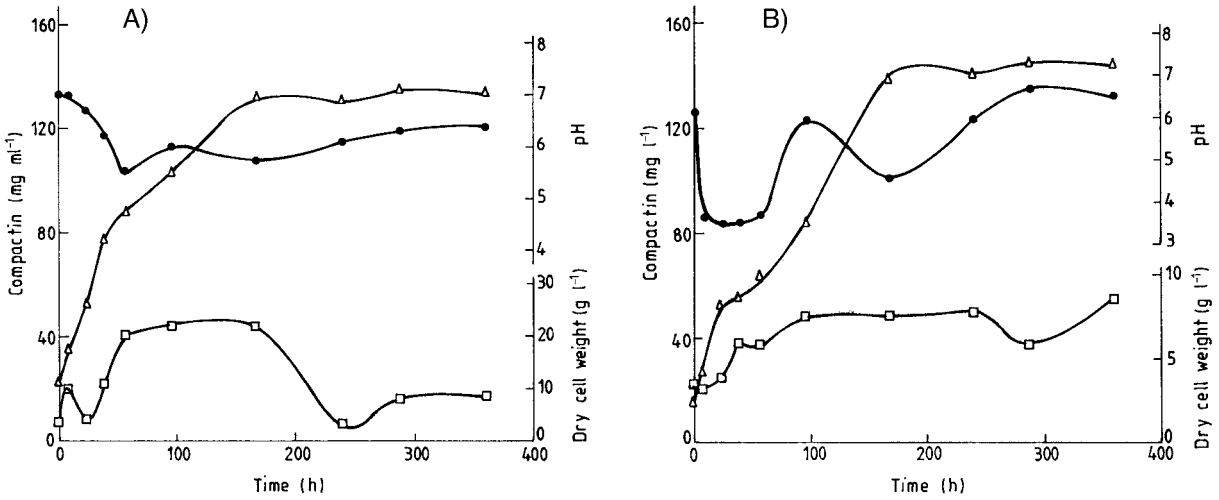

Fig. 1. Fermentation profiles of compactin (△), dry cell weight (□) and pH (●) during the cultivation of *P. citrinum* in A: PRC-A medium (glucose, 1 g l^{-1}; maltose, 1 g l^{-1}; glycerol, 3.5 g l^{-1}; urea, 0.54 g l^{-1}; soya oil, 20 ml l^{-1} and $MgSO_4$ 0.05 g l^{-1}); B: PRC-B medium (glucose, 2 g l^{-1}; maltose, 2 g l^{-1}; glycerol, 13.5 g l^{-1}; urea, 0.54 g l^{-1} and $MgSO_4$, 0.05 g l^{-1}). Each medium was inoculated at 5% (v/v) with 2-d old IM-A culture and incubated at 24 °C and 220 rpm. Compactin was measured spectrophotometrically and the presented data are the average of triplicate determinations, where the individual values were within ± 10% of the average. All the experiments were performed in triplicate.

7.5 g l^{-1} and $MgSO_4$, 1 g l^{-1}). Media were sterilized at 121 °C for 20 min. The pH of these media, prior to sterilization, was 6.3–6.4. The three different production media (PRC) [PRC-A, PRC-B and PRC-C (composition of these media are mentioned in the legend of Figure 1)] were used in this study. The constituents of PRC-C medium were same as that of PRC-A medium excepting urea, which was replaced by both $(NH)_2SO_4$, 0.12 g l^{-1} and NH_4Cl, 0.049 g l^{-1}. All three media were sterilized at 121 °C for 20 min. Before inoculation, the pH of these media was adjusted to 6.5 with 2 M H_3PO_4. All media components were obtained from Hi Media Ltd. Compactin of 99.9 % purity was obtained from Sigma Chemicals.

Fermentation

All three inoculum media (50 ml medium in 250 ml Erlenmeyer flask) were inoculated with the spore suspension at 1% (v/v) and incubated at 220 rpm and 28 °C for 2 d. Production media (200 ml medium in 1000 ml Erlenmeyer flask) were inoculated with inocula at 5% (v/v) and incubated at 24 °C at 220 rpm in orbital shaker for 10–15 d. Samples were aseptically withdrawn at intervals for analysis of compactin, dry cell weight and pH. The effect of number of spores used for inoculation (10^2–10^8 spores ml^{-1}) in inoculum medium and initial pH (4.5–7) of production medium, on the yield of compactin in the most suitable

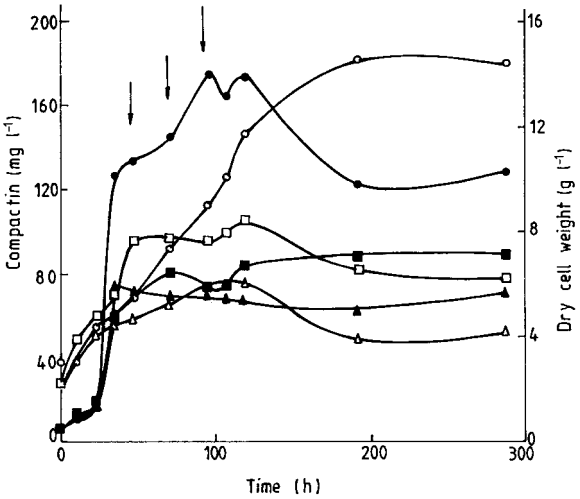

Fig. 2. Effect of addition of Tween 80 on the compactin and dry cell weight profiles during cultivation of *P. citrinum* in PRC-A medium (→ shows time of addition of Tween 80 [Compactin: ▲ control (no addition of Tween 80); ■ with Tween 80 added at the beginning at 5 g l^{-1}; and ● Tween 80 added at 1 g l^{-1} after 48, 72 and 96 h. Dry cell weight: △ control (no addition of Tween 80); □ Tween 80 added at the beginning at 5 g l^{-1}, ○ with Tween 80 added at 1 g l^{-1} on 48, 72 and 96 h.] The PRC-A medium was inoculated at 5 % (v/v) with 2-d old IM-A culture and incubated at 24 °C and 220 rpm. Compactin was estimated spectrophotometrically and the data presented are the average of triplicate determinations, where the individual values were within ± 10% of the average. All the experiments were performed in triplicate.

production medium, was also studied. The initial pH of production medium was adjusted after sterilization with either 2 M H_3PO_4 or 2 M NaOH. All experiments were performed thrice in triplicate. The data presented are the average of triplicate determinations, where the individual values were within ±10% of the average.

Analysis

The number of spores in the spore suspension were counted using a hemocytometer after vortexing and appropriate dilution. Dry cell weight was estimated by filtering a known volume of culture broth through pre-weighed Whatman paper no. 41 and drying the paper at 60 °C under partial vacuum for 10–12 h. Filter papers were then weighed repeatedly after regular intervals to a constant weight.

For compactin estimation, samples were adjusted to pH 6.5 by either 4 M H_3PO_4 or 2 M NaOH, diluted to 5 times with absolute ethanol, filtered through Whatman filter paper No. 41 and the absorbancy was monitored at 238 nm. From the standard curve of compactin in absolute ethanol, in which compactin was analysed by HPLC (Konya *et al.* 1998) and spectrophotometrically, the following relation was obtained:

$$C_H = 0.0148A,$$

where C_H is the concentration of compactin (mg ml^{-1}) as measured by HPLC and A is absorbancy at 238 nm. As the components of the production media did not show any interference at 238 nm, compactin estimation in all the experiments was done by the spectrophotometric method using the above relation. Compactin in the fermentation broth was stable and did not undergo any interaction with other metabolites present in the fermentation broth. The compactin concentration in various samples (stored at 4 °C) did not change for at least 15 d. However, the maximum variation in the compactin concentration when estimated in various fermentation samples by both spectrophotometric and HPLC method, was only ±4%.

Results and discussion

Among the three inoculum media, only IM-A supported the growth of *P. citrinum* and it was therefore used for further studies.

The use of defined medium at production stage resulted in an easier method of compactin estimation as compared to other methods (Hamdy *et al.* 1998, Endo *et al.* 1976a,b, 1985, 1986, Hosobuchi *et al.* 1993a,b). However, in the samples up to 24 h, there was discrepancy in the compactin values estimated by spectrophotometric method. This was due to the presence of complex nitrogen sources that entered the production media via the inoculum.

Production of compactin was faster and higher in the PRC-A and PRC-B medium at 135 mg l^{-1} and 145 mg l^{-1}, respectively, after 288 h irrespective of the presence of oil in the medium (Figures 1A and 1B). In the PRC-C medium, the maximum compactin production was only 65 mg l^{-1} at 288 h. The irregular pH profile of PRC-B culture was improved by inclusion of buffering agent, e.g. KH_2PO_4, in the medium at 1.5 g l^{-1} but without increasing compactin production.

Effect of Tween 80

Oil present in PRC-A and PRC-C media interfered with the dry cell weight estimation. To remove this interference, Tween 80 was added either at the beginning (5 g l^{-1}) or at intervals (1 g l^{-1} at 48 h, 72 h and 96 h) during fermentation. The later regimen led to higher accumulation of compactin (175 mg l^{-1}) and biomass (14.5 g l^{-1}) than when Tween 80 was either not added (control) or added at the beginning (Figure 2). Similar results were obtained with PRC-C medium (data not shown).

The increase in compactin production could be explained by dispersion of the oil by surfactant (Hosobuchi *et al.* 1993a).

Effect of spore concentration and initial pH

Tween 80 present in the fermentation medium tends to produce foam during the fermentations. PRC-B medium was chosen for future studies because it did not contain soya oil and therefore did not require Tween 80 addition, necessary for removal of interference in dry cell weight estimation. Using this medium, different numbers of spores were inoculated and the spore concentration of 10^7 ml^{-1} produced the best result in terms of compactin (150 mg l^{-1}) and it also resulted in pelleted culture. Use of higher or lower spore concentrations decreased the compactin production. Maximum compactin (145 mg l^{-1}) was obtained when the initial pH of the production medium was 6.5–7, whereas minimum compactin production (35 mg l^{-1}) was with an initial pH of 4.5.

Conclusions

A maximum of 175 mg compactin l^{-1} was obtained in 96 h by using defined medium at the production stage. An easy spectrophotometric method for compactin estimation was also designed. To the best of our knowledge, this is the first report where defined medium was used at the production stage for compactin production.

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