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Residual fructose and osmolality affect the levels of pneumocandins B_0 and C_0 produced by *Glarea lozoyensis*

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Abstract A high total pneumocandin titer (B_0 + C_0) with a low percentage of the structural isomer pneumocandin C_0 was achieved by carrying out fermentations of *Glarea lozoyensis* at a high residual fructose concentration (125 g/l initial). When the fermentation was carried out at a low residual fructose concentration (40 g/l initial), pneumocandin production increased by 34%. However, a disproportionate increase in the level of pneumocandin C_0 synthesized (250% increase vs 30% increase for pneumocandin B_0) was observed. Midcycle addition of 150 mM NaCl or 116 mM Na_2SO_4 to low residual fructose fermentations returned the titer and isomer levels to those seen for the high residual fructose fermentation. The increase in pneumocandin C_0 synthesis under low residual fructose conditions appears to be linked to the increase in the synthesis of *trans*-4 hydroxyproline, with the synthesis of *trans*-3 hydroxyproline remaining unaffected. This suggests that the formation of pneumocandin C_0 is the result of a misincorporation of *trans*-4 hydroxyproline instead of *trans*-3 hydroxyproline by the pneumocandin peptide synthetase, and that the amount of *trans*-4 hydroxyproline formed dictates the frequency of this misincorporation.

Introduction

The fungus *Glarea lozoyensis* (previously identified as *Zalerion arboricola*, see Bills et al. 1999) produces a family of structurally related acylated cyclic hexapeptides known as the pneumocandins (Schwartz et al. 1992; Hensens et al. 1992). Semisynthetic derivatives of the natural product of interest, pneumocandin B_0 (Fig. 1), are potent inhibitors of β-(1,3)-glucan synthesis and possess an excellent spectrum of fungicidal activity against clinically important isolates while remaining nonhemolytic for human and mouse erythrocytes (Bartizal et al. 1995; Del Porta et al. 1997).

Pneumocandin B_0, presumably synthesized by a peptide synthetase, consists of the amino acids threonine, *trans*-4 hydroxyproline, 3,4-dihydroxyhomotyrosine, 3-hydroxyglutamine, *trans*-3 hydroxyproline, and 4,5-dihydroxyornithine in addition to a 10,12-dimethylmyristate side chain (Fig. 1). Pneumocandin C_0 is a structural isomer of pneumocandin B_0 where a *trans*-4 hydroxyproline residue is misincorporated in place of the *trans*-3 hydroxyproline found in pneumocandin B_0 (Fig. 1). Large-scale silica-gel chromatography can resolve the two pneumocandins (Osawa et al. 1999). However, a better understanding of the factors controlling pneumocandin C_0 formation would allow the level of this structural isomer to be controlled in the fermentation rather than relying completely on downstream processing steps.

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Materials and methods

Pneumocandin fermentation

The *Glarea lozoyensis* strain used in this study is a descendant of ATCC 74030 generated through a classical mutation/strain improvement program. The culture was maintained as aliquots of a mycelial suspension in 5% (v/v) glycerol at −70 °C. LYCP-5 medium was used for inoculum development and consisted of (per liter): glucose, 25 g (added after sterilization); KH_2PO_4, 9 g; yeast extract (Difco, Detroit, Mich., USA), 5 g; cotton seed flour (Traders Protein, Memphis, Tenn., USA), 10 g, lactic acid (85%), 2 ml; and a trace element mixture, 10 ml. The trace element mixture consisted of (per liter): $FeSO_4 \cdot 7H_2O$, 1.0 g; $MnSO_4 \cdot H_2O$, 1.0 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; HBO_3, 0.056 g; $CuCl_2 \cdot 2H_2O$, 0.025 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.019 g; and 12 N HCl, 50 ml. The presterile pH of the LYCP-5 medium was adjusted to 6.0. FGY medium was developed to support pneumocandin production at the shake-flask and 23-l fermentor scales and consisted of (per liter): fructose, 40 or 125 g (added after sterilization);

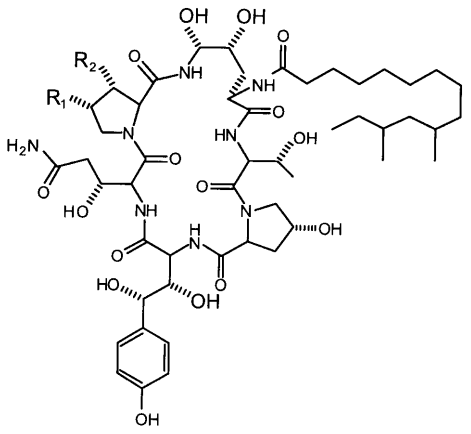

Fig. 1 Structures of pneumocandin B_0 ($R_1 = H$, $R_2 = OH$) and pneumocandin C_0 ($R_1 = OH$, $R_2 = H$)

monosodium glutamate, 8 g; yeast extract (BioSpringer, Minneapolis, Minn., USA), 8 g; proline, 15 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; and trace element mixture, 10 ml. The pH was adjusted to 5.3 prior to sterilization. For shake-flask experiments, the FGY medium was supplemented with 15 g/l 2-[*N*-morpholino]ethanesulfonic acid buffer.

The fermentation process consisted of two stages of inoculum development and a shake-flask or 23-l fermentor production stage. A first-stage inoculum was generated by inoculating 1 ml frozen mycelia into 50 ml LYCP-5 medium in a 250-ml Erlenmeyer flask. The culture was incubated for 4 days at 25 °C with 220 rpm rotary shaking. The second-stage inoculum for shake-flask experiments was generated by inoculating one or more 250-ml Erlenmeyer flask(s) containing 50 ml LYCP-5 with 1 ml first-stage inoculum and incubating as above for 3 days. The second-stage inoculum for 23-l fermentor experiments was generated by inoculating one or more 2-l Erlenmeyer flask(s) containing 500 ml LYCP-5 medium with 10 ml of the first-stage inoculum and incubating for 3 days.

For production fermentations at the shake-flask scale, a 300-ml aliquot of sterile FGY medium was inoculated with 15 ml second-stage seed followed by subdivision at 25 ml per sterile 250-ml Erlenmeyer flask. This procedure improved the precision for each treatment group within an experiment. The cultures were incubated at 25 °C with 220 rpm rotary shaking. Beginning on or about day 6 of the production cycle, one flask was harvested daily from each treatment group to allow pH adjustment (5.3–5.5) of the remaining flasks using sterile titrant. Titers were normalized to the initial flask volume as determined by mass change. Shake-flask fermentations were typically carried out for 14 days, and the data presented for a given treatment group represent duplicate flasks harvested on this day. Results are representative of replicate experiments. Alterations or additions to the base medium are described in the text.

For experiments in laboratory fermentors, 23-l scale fermentors (Chemap Inc., South Plainfield, NJ, USA) containing 15 l sterile FGY medium were inoculated with 500 ml second-stage inoculum. Initial conditions for the fermentation were: temperature, 25 °C; agitation, 300 rpm; aeration, 0.4 vvm; backpressure, 0.5 kg/cm^2; pH 5.4 was maintained with 10% (v/v) sulfuric acid and 10% (w/v) sodium hydroxide. The dissolved oxygen was kept above 30% of saturation by controlling agitator speed (300–750 rpm). Data presented are representative of replicate experiments.

To maintain a high residual fructose level during the course of the production cycle (> 90 g/l), the initial sugar concentration was 125 g/l and 50 g/l (final concentration) additions were made on days 7 and 11. To maintain a low residual fructose level (< 35 g/l), the initial sugar concentration was 40 g/l and 25-g/l (final concentration) additions were made every other day beginning on day 4. Where indicated, the addition of NaCl or Na_2SO_4 was made on day 4 to a final concentration of 150 or 116 mM, respectively.

Pneumocandin extraction and quantitation

Reverse-phase chromatography was used to determine the total pneumocandin titer (i.e., $B_0 + C_0$) and normal phase chromatography was used to determine the percentage of pneumocandin C_0 in the sample. For the determination of the pneumocandin $B_0 + C_0$ titer by reverse-phase chromatography, an aliquot of whole broth was mixed with three volumes of 100% methanol, homogenized with a tissue homogenizer, and mixed for 60 min. The methanol extract was centrifuged at 1500*g* for 10 min to remove cell debris. Clarified methanol extracts were filtered through a 0.45-μm PVDF syringe filter prior to analysis. Ten microliters of the methanol extract were chromatographed on a YMC J'Sphere ODS-M80 (YMC Inc., Wilmington, N.C., USA) column at 30 °C using a gradient method. With a flow rate of 1.5 ml/min, the acetonitrile:0.1% (v/v) phosphoric acid in water ratio was maintained at 40:60 for the first 20 min (after injection) and then increased to 50:50 between 20 and 40 min. Ultraviolet absorption of the eluant was monitored at 210 nm. The pneumocandin $B_0 + C_0$ titer was determined based on calibration of the instrument with a pneumocandin B_0 standard of known purity.

To determine the ratio of pneumocandin B_0 to C_0 by normal phase analysis, equal volumes of whole broth and isobutanol were mixed for 60 min. The mixture was centrifuged as above to remove cell debris and separate the aqueous and organic phases. A 10- to 15-ml aliquot of the organic phase was evaporated to dryness and the residue was dissolved in 2 ml mobile phase (ethyl acetate:methanol:water, 84:9:7) and filtered prior to analysis. Twenty microliters of the reconstituted extract were chromatographed on an Inertsil 5 μ SI column (MetaChem Technologies, Torrance, Calif., USA) at ambient temperature employing a mobile phase of ethyl acetate:methanol:water (84:9:7) at a flow rate of 1.2 ml/min. Ultraviolet absorption of the eluant at 278 nm was monitored and pneumocandins B_0 and C_0 were identified by comparison to pure standards. The level of pneumocandin C_0 was expressed as a percentage of the total amount of pneumocandin $B_0 + C_0$ based on area counts.

Analytical methods

To determine the levels of hydroxyprolines produced during the fermentation, an aliquot of whole broth was boiled for 5 min followed by centrifugation to remove biomass and insolubles. Quantitation was carried out on the supernatant using the Hewlett Packard AminoQuant system (Hewlett Packard, Palo Alto, Calif., USA). Concentrations presented in the Results section were normalized to biomass in the original sample that was measured by dry cell weight.

Osmolality was measured using a vapor pressure osmometer (Wescor, Inc., Logan, Utah, USA). Standard solutions of NaCl were used for calibration and values were expressed in millimoles per kilogram, consistent with IUPAC convention.

Chemicals, media components, and standards

All chemicals and media components, unless otherwise noted, were purchased from Sigma (St. Louis, Mo., USA) and were reagent grade. High purity standards of the pneumocandins were generated internally as part of an ongoing purification development effort.

Results

Maintaining a high residual fructose concentration (i.e., 125 g/l initial fructose) gave acceptable pneumocandin titers (i.e., $B_0 + C_0$) and afforded control over the synthesis of pneumocandin C_0 (Table 1). In this context, "acceptable" is defined as the titer which provides a

Table 1 Effects of "high" vs "low" residual fructose concentrations

	"High fructose" (125 g/l initial)	"Low fructose" (40 g/l initial)
$B_0 + C_0$ titer[a]	253	339
C_0 percentage	4.3	8.1
Adjusted B_0 titer	242	312
Adjusted C_0 titer	11	27

[a] Titer is given in arbitrary units and is the average of duplicate flasks assayed at 14 days

significant economic return. The overall pneumocandin titer (i.e., pneumocandins $B_0 + C_0$) was increased by 34% when the process was run at low residual fructose levels. The overall titer increase was the result of a 30% increase in the synthesis of B_0 (i.e., adjusted B_0 titer) and a 250% increase in the synthesis of C_0 (i.e., adjusted C_0 titer) (Table 1). The disproportionate increase in the amounts of pneumocandins B_0 and C_0 resulted in a doubling of the pneumocandin C_0 percentage. While the increase in the pneumocandin B_0 titer was desirable, implementation of this process change was unacceptable due to the increase in the level of pneumocandin C_0, which would have furthered challenged the silica-gel chromatography step (Osawa et al. 1999).

The osmolality for the "high fructose" process (i.e., 125 g/l initial fructose) was approximately 1000 mmol/kg, while the osmolality for the "low fructose" process (i.e., 40 g/l initial fructose) was 650 mmol/kg. The osmolality seen with the "high fructose" process could be regained by adding 150 mM NaCl or 116 mM Na_2SO_4 to the "low fructose" process on day 4, resulting in measured osmolalities of 925 and 980 mmol/kg respectively. The addition of the salts as "osmotic buffers" reduced the pneumocandin titers closer to those typically seen with the "high fructose" process (Table 2). Moreover, the level of pneumocandin C_0 produced was reduced to levels more amenable to removal by chromatography. Addition of salts to the "high fructose" process resulted in a significant reduction in titer (data not shown).

The use of NaCl as an osmotic buffer was evaluated at the 23-l fermentor scale by running fermentations at a low residual fructose level with and without 150 mM NaCl added on day 4. Fermentations run at low residual fructose with and without added salt produced pneumocandins at comparable rates with similar final titers (Fig. 2). These results were in contrast to those obtained

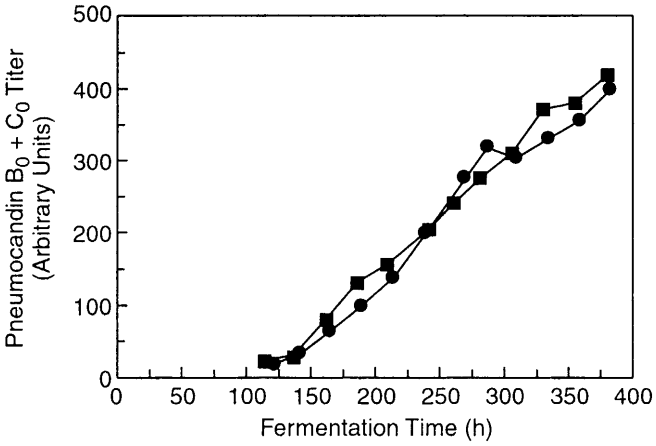

Fig. 2 Pneumocandin titer ($B_0 + C_0$) for the "low fructose" process run at the 23-l fermentor scale with (■) or without (●) 150 mM NaCl added on day 4

at the shake scale, where addition of salt resulted in a reduction of the titer (Table 2). This observation may be attributable to differences in scale or to some other unidentified parameter. A comparison of the levels of pneumocandin C_0 produced revealed that for the batch run without added salt, the percentage of pneumocandin C_0 was between 8 and 10% up to 300 h and then increased sharply to over 15% at the end of the cycle (≈375 h; Fig. 3). In contrast, the pneumocandin C_0 percentage for the batch receiving the salt addition

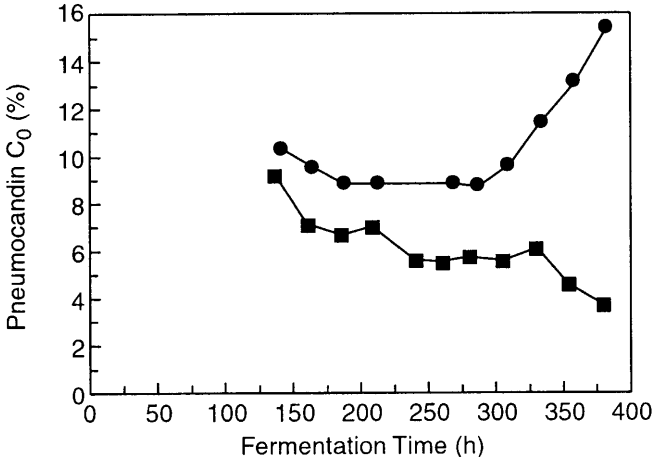

Fig. 3 Level of pneumocandin C_0 produced in the "low fructose" process run at the 23-l fermentor scale with (■) or without (●) 150 mM NaCl added on day 4

Table 2 "Low fructose" process with and without added salts for osmotic buffering

	"High fructose" (125 g/l)	"Low fructose" (40 g/l)	"Low fructose" +150 mM NaCl	"Low fructose" +116 mM Na_2SO_4
$B_0 + C_0$ titer[a]	253	339	282	280
C_0 percentage	4.3	8.1	5.5	6.2
Adjusted B_0 titer	242	312	266	263
Adjusted C_0 titer	11	27	16	17

[a] Titer is given in arbitrary units and is the average of duplicate flasks assayed at 14 days

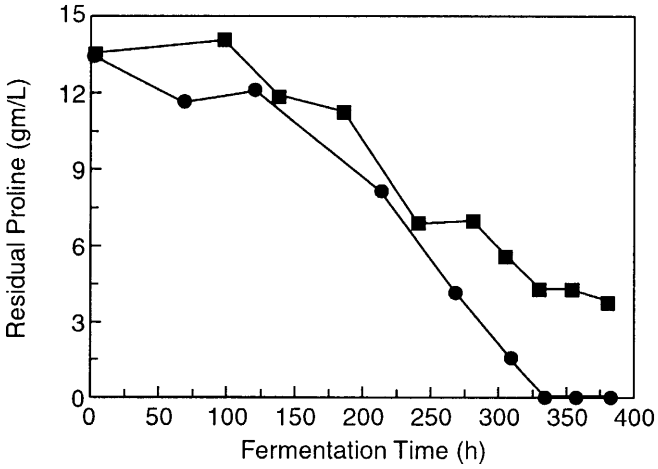

Fig. 4 Kinetics of proline utilization in the "low fructose" process run at the 23-l fermentor scale with (■) or without (●) 150 mM NaCl added on day 4

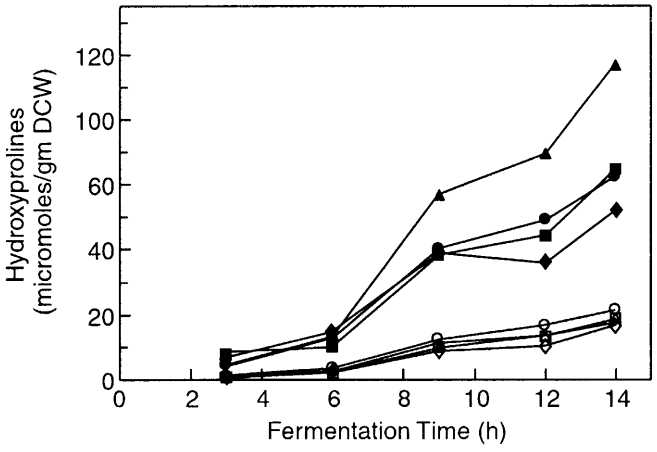

Fig. 5 Production of *trans*-4 hydroxyproline (*filled symbols*) and *trans*-3 hydroxyproline (*open symbols*) at the shake-flask scale under different osmotic conditions. "High fructose" process (○,●); "low fructose" process (▲,△); "low fructose" plus 150 mM NaCl added on day 4 (■,□); "low fructose" plus 116 mM Na_2SO_4 added on day 4 (◆,◇)

decreased from approximately 8% to under 5% by the end of the cycle (Fig. 3).

When the residual proline levels for the two batches were compared, it was apparent that the extent of proline utilization was higher for the batch that did not receive the salt addition (Fig. 4). Biomass accumulation was similar for both batches, so this does not account for the difference in proline consumption (data not shown). The dramatic increase in the pneumocandin C_0 percentage at around 300 h corresponds to the depletion of proline. This result is consistent with previous findings which identified the addition of proline as key to controlling the level of pneumocandin C_0. However, it is important to note that the batch run without salt addition produced a consistently higher percentage of pneumocandin C_0 even when the residual proline concentration was between 5 and 10 g/l (e.g., between 150 and 250 h).

Figure 5 illustrates the underlying mechanism of how changes in osmolality affect the production of *trans*-3 and *trans*-4 hydroxyproline. When the pneumocandin production fermentation is run at a low residual fructose concentration, the specific production of *trans*-4 hydroxyproline is almost double that of the fermentation run at a high residual fructose concentration. When NaCl at 150 mM or Na_2SO_4 at 116 mM is added to the low residual fructose process, the amount of *trans*-4 hydroxyproline formed is similar to the levels seen for the high residual fructose process. Furthermore, none of these fermentation conditions affects the levels of *trans*-3 hydroxyproline formed. These data lead to the conclusion that the increase in the formation of pneumocandin C_0 is a result of the increase in the amount of *trans*-4 hydroxyproline formed rather than a decrease in the formation of *trans*-3 hydroxyproline. Moreover, the ratio of the amounts of hydroxyprolines formed is essential for determining the percentage of pneumocandin C_0 produced.

Discussion

In a study carried out with *Streptomyces clavuligerus*, glucose, sorbitol, and NaCl were used as water activity depressors. β-Lactam antibiotic production was affected when a_w (water activity) was reduced below 0.997. However, growth was not impacted dramatically until a_w was reduced to 0.990 (Cochet and Demain 1996). Extracellular γ-decalactone production, an aroma metabolite produced by the yeast *Sporidiobolus salmonicolor*, was increased at a water activity of 0.99 and release of intracellular γ-decalactone occurred at 0.94 and 0.97 (Gervais and Battut 1989). Reduced water activity in combination with glucose additions altered the expression of the pectinases and protease produced by *Aspergillus niger*, with a synergistic stimulation of polygalacturonase production being observed at lower water activity in the presence of added glucose (Taragano et al. 1997).

In this study, we demonstrated not only how the titer of a secondary metabolite can be affected by available water but also how this parameter can be manipulated to control the metabolite spectrum. In the case of pneumocandin production by *Glarea lozoyensis*, an acceptable pneumocandin $B_0 + C_0$ titer and structural isomer control was obtained at a high osmolality (900–1000 mmol/kg, reduced available water) by carrying out the fermentation either at a high residual fructose concentration (125 g/l initial) or at a low residual fructose concentration (40 g/l initial) with added salt (150 mM NaCl or 116 mM Na_2SO_4). These results are akin to those obtained by Baxter et al., who demonstrated that the production of squalestatins S1 and S2 were differentially affected by water activity and temperature combinations (Baxter et al. 1998).

Carrying out the fermentation at a low residual fructose concentration (osmolality of 650 mmol/kg) resulted in a disproportionate increase in the production of pneumocandin C_0. The mechanism for this appears to be an increase in the synthesis of *trans*-4 hydroxyproline which is then misincorporated during the synthesis of the hexapeptide. It is not clear how the synthesis of *trans*-4 hydroxyproline is regulated by osmolality or why the synthesis of *trans*-3 hydroxyproline is unaffected by this parameter. This result does suggest, but does not conclusively prove, that the regiospecific hydroxylation of proline is the result of two differentially regulated proline hydroxylase activities.

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