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Early Phase Process Scale-Up Challenges for Fungal and Filamentous Bacterial Cultures

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Abstract

Culture pelleting and morphology has a strong influence on process productivity and success for fungal and filamentous bacterial cultures. This impact is particularly evident with early phase secondary metabolite processes with limited process definition. A compilation of factors affecting filamentous or pelleting morphology described in the literature indicates potential leads for developing process-specific control methodologies. An evaluation of the factors mediating citric acid production is one example of an industrially important application of these techniques. For five model fungal and filamentous bacterial processes in an industrial fermentation pilot plant, process development strategies were developed and effectively implemented with the goal of achieving reasonable fermentation titers early in the process development cycle. Examples of approaches included the use of additives to minimize pelleting in inoculum shake flasks, the use of large-volume frozen bagged inoculum obtained from agitated seed fermentors, and variations in production medium composition and fermentor operating conditions. Results were evaluated with respect to productivity of desired secondary metabolites as well as process scalability. On-line measurements were utilized to indirectly evaluate the cultivation impact of changes in medium and process development. Key laboratory to pilot plant scale-up issues also were identified and often addressed in subsequent cultivations.

Index Entries: Morphology; pellet; fermentation; scale-up; *Aspergillus*; *Sordaria*; *Streptomyces*; *Chalara*.

Introduction

Because culture morphology often affects fermentation productivity and impacts successful scale-up, process-specific methods to influence and

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control this parameter need to be developed *(1)*. Formation of pellets in seed and production cultivations of fungal cultures has been described and studied extensively. The initial goal is to determine what type of morphology (from highly filamentous to highly pelleted) is best for productivity. This analysis then is followed by process development aimed at reliably obtaining the desired culture form. Pellet formation can occur in any fermentation stage from the frozen inoculum vial to the production fermentor. Although desirability and acceptability of pellets in each stage depends heavily on the specific process and its stage of process development, some general statements typically hold true.

Inoculum characteristics definitely affect production fermentor performance *(2)*. Pellets present a problem for inoculum because they cannot be pipeted uniformly into master/working seed vials or the next flask stage. In addition, they may clog peristaltic tubing used to transfer the shake-flask contents to the seed fermentor. Inoculum pellet formation can be avoided by using a Waring blender or hand mixer *(3)* to homogenize the seed (which has extensive drawbacks for subsequent culture purity), by switching to baffled shake flasks (which may cause excessive foam formation), by adding glass beads *(4,5)* to break up clumps (which is limited by the shaking rate because beads may break glass flasks), by using spores cultured for several days on a slant (which requires additional time for sporulation), and by adding agar to shake-flask seed medium (which is limited by agar solubility). Pellets present in early stages of an inoculum train often increase in size during later stages. Extending the inoculum stage incubation period causes larger pellets for some cultures and a transition from pelleting to mycelial growth in other instances.

Pellets present a problem in fermentor cultivation because growth is slower (owing to the reduced number of active growth centers), less reproducible, and subject to dissolved oxygen (DO) limitation close to the pellet core. Representative culture samples often are difficult to obtain because pellets quickly settle in nonagitated broth samples. Growth of molds in pellet form can be an advantage in some industrial fermentation processes *(6)*, particularly when it results in lower broth viscosity and thus decreased power input requirements for proper mixing and aeration *(7)*.

The purpose of this article is to summarize and categorize literature references describing external influences on pelleting and culture morphology. Production of citric acid by *Aspergillus niger* is then evaluated based on literature references as one example of fermentation in which morphology is compared with other parameters for its influence on productivity. Along with other scale-up techniques, some of the major factors influencing morphology were applied to the successful initial pilot-scale cultivations of several fungal (*A. niger*, *Aspergillus unguis*, *Sordaria araneosa*, *Chalara affinis*) and filamentous bacterial (*Streptomyces roseoflavus*) cultures during early phase process development efforts. Often at this early stage, secondary metabolite synthesis pathways are less studied and few reports are available about the scale-up of these strains. Additional issues, not

related to culture morphology, associated with pilot scale-up based on laboratory-scale fermentations are identified and described.

Culture Pelleting and Influences on Morphology

Several key articles exist summarizing pelleting and general morphology. A general article that focuses on *Aspergillus* has an excellent discussion and analysis of factors that influence pellet formation based on the literature available at the time it was written *(6)*. Another article provides a thorough compilation of references with descriptions of contemporary research in several areas related to pelleting, again based on literature available at the time *(8)*. Two additional articles each have an extensive list of *Actinomycetes* organisms forming either pellets or mycelia exclusively and those exhibiting both morphologies in shake flasks and in stirred vessels *(9,10)*. Finally, a comprehensive review article on fungal morphology was recently published *(11)*.

A brief summary of the general effects on pelleting vs filamentous growth discussed in the literature is provided in Tables 1–5, with the specifics of cultivation conditions listed where available. Areas covered are agitation, inoculum, media composition, media additives, and fermentation parameters. Studies involving agitation have compiled data relating mean hyphal length to power input for various fungi *(12)* and developed scale-up equations based on impeller type and agitation intensity *(3,13,14)*.

Influences on Pellet Formation

There are three major classes of variables that can be controlled to influence pellet formation, assuming that media composition, strain, and organism remain constant: inoculum concentration, addition of polymer additive, and shear forces in the fermentor *(12)*.

Pellets generally are formed at inoculum levels below 10^5 spores/mL (10^{11} spores/m^3), and, above this concentration, dispersed filamentous growth typically prevails *(12,15,16)*. An optical density at 600 nm of 0.5 U is equivalent to 10^6 spores/mL of *A. niger*, a relationship that reduces tedious enumeration assays prior to inoculation *(17)*. The inoculum character can influence eventual production culture morphology, which, in turn, influences productivity *(18)*. Specifically, inoculation with spores (conidia) was preferred over inoculation with mycelia for growth of *A. niger* to produce pectic enzymes *(19)*.

Polymer additives, typically nonionic and anionic surfactants, affect pellet formation by influencing spore agglomeration in a concentration-dependent manner *(20,21)*. Reduced spore aggregation promotes dispersed filamentous growth, which results in higher culture growth and production rates, particularly for enzyme production processes *(22)*.

In general, if the culture is not shear sensitive and power input is low, one pellet forms from one spore *(16)*. If the culture is shear sensitive, more than one pellet can form from each spore owing to pellet fragmentation.

Table 1
Effects of Agitation on Pelleted vs Filamentous Growth

Agitation effect	Impact	Specific example
Higher agitation rate	Smaller pellets, eventually filamentous growth	Shorter hyphal lengths from 1300 μ at 400 rpm to 800–1000 μ at higher rates (550, 700, and 1100 rpm), earlier fragmentation at higher speeds, no real evaluation of effect on pellet diameter, 10^6 spores/mL inoculum, 7- to 8-L fermentation, *Aspergillus awamori (61)*
	Smaller clumps with shorter and thicker filaments	320 μ for 200 rpm, 240 μ for 400 rpm, 190 μ for 600 rpm; for 200-rpm case increase in size of clumps over course of fermentation; biomass not affected; 8-L fermentation; *A. niger (62)*
	Decrease in pellet diameter	35 μ for >550 rpm, 60 μ for 400 rpm, 375 μ for 250 rpm, 1.5-L fermentation, *Streptomyces frandiae (63)*
	Pellets then mycelial growth	Above 300 rpm, formation of 2- to 3-mm pellets within 24–48 h, which were later crushed into homogeneous mycelial dispersion, 2.7×10^5 spores/mL inoculum, 12-L fermentor, *A. niger (41)*
		Pellets of about 2 mm at 91 rpm and filamentous growth at 155 rpm, 1×10^5 spores/mL inoculum, 500-L fermentor, *A. niger (64)*
Higher tip speed	No change in morphology	No change for tip speed from 2.4 to 3.6 m/s, $0.1–5 \times 10^5$ spores/mL inoculum, 2-L fermentor, *Penicillium chrysogenum (65)*
Higher shear rate	Decrease in pellet diameter	In 300-mL spinner flasks agitated with paddle, linear decrease in pellet diameter with shear rate for rates <400 s^{-1}; above 400 s^{-1} pellet diameter constant; 1×10^5 spores/mL inoculum; *A. niger (17)*
Higher shear rate and shear stress	Change in morphology from pelleted to filamentous	Change in morphology for shear stress >5 Pa; as shear rate increased to 720 s^{-1} filaments more branched with improved mechanical resistance; used annular reactor, *A. niger (66)*
Higher shear force in inoculum stage	Narrower pellet distribution and decrease in pellet size	Decrease from 1 to 0.6 mm in production culture owing to smaller pellets in inoculum obtained by varying number of shake-flask baffles, shaker speed, and amount of glass spheres; 100-L fermentor; 2×10^6 spores/mL inoculum; *P. chrysogenum (4)*
Higher energy dissipation rate	Decrease in pellet size	Diameter proportional to (dissipation rate)$^{-0.16}$, $10^3–10^4$ spores/mL inoculum, 2-L fermentation, *A. awamori (23,67)*
Switch from countercurrent impeller type with lower shear to turbine with higher shear	Decrease in pellet size	Pellets denser with smaller annular region, higher broth mycelial fraction and higher viscosity, 6-L fermentation, 3.5×10^5 spores/mL inoculum, *A. niger (14)*

Table 2
Effects of Inoculum on Pelleted vs Filamentous Growth

Inoculum effect	Impact	Specific example
Higher spore concentration of inoculum	Smaller pellets, eventually filamentous growth	10^4 vs 10^5 vs 10^6 spores/mL: shorter time to reach peak biomass by 5–6 h per order of magnitude jump; number of tips per hyphal element lower: 12 vs 10 vs 8 (2 less tips per order of magnitude jump); no real evaluation of effect on pellet diameter; 7- to 8-L fermentation; *A. awamori (61)*
	Clumps less compact but rougher, smaller in diameter	Pellets of equivalent diameter of 300 μ with use of 10^4 spores/mL, and pellets of equivalent diameter of 100 μ with use of 10^5 spores/mL; clumps threefold less compact and sixfold rougher; shake flasks; *P. chrysogenum (68)*; effect not as pronounced in fermentors as in shake flasks *(15)*; decrease in fermentor pellet size from 180 to 125 μ when spore inoculum concentration increased from 1 to 5×10^4 spores/mL *(65)*
	Pelleted growth then mycelial then pelleted form	Pellets with use of 2.2×10^4 spores/mL, dispersed mycelia/loose clumps with use of 4.5×10^5 to 1.7×10^6 spores/mL, and pellets with use of 5×10^6 to 1.1×10^7; 0.5-L fermentation; *Streptomyces clavuligerus (69)*
	Change in pelleted form from dense to open and decreased in size	Dense amorphous center with short hyphae with use of 5×10^2 spores/mL (0.9 mm in diameter) to 1×10^3 spores/mL (0.7 mm in diameter); more open pellet with no more than single growth point at center with use of 1×10^4 spores/mL (0.4 mm in diameter), 1×10^5 spores/mL (0.2 mm in diameter), and 1×10^6 spores/mL (0.1 mm in diameter); shake flasks; *P. chrysogenum (18)*
	Decrease in pellet size and then turned into mycelial growth	Nonbaffled shake flasks: <1 spore/mL: pellet >2 mm; 10–10^4 spores/mL: pellet ~1 mm; >10^4 spores/mL: pellet reduced to 0.5 mm; baffled shake flasks (smaller pellets): <1 spore/mL: pellets ~1 mm; 10–10^4 spores/mL: pellet 0.7–0.8 mm; >10^4 spores/mL: branched mycelial growth; shake flasks; *Streptomyces tendae (70)*
Use of spore inoculum	Pulpy mycelium with loose pellets	Inoculum of 1.2×10^7 spores/mL, 3-L fermentation, *A. niger (71)*
Use of pelleted inoculum	Compact, smooth pellets	Inoculum of 9.8×10^2 pellets/mL without spores, 3-L fermentation, *A. niger (71)*
Higher pellet concentration in inoculum	Increase in pellet size	Initial pellet size of 0.8 mm; after 24–48 h; growth of pellet inoculum of 11 pellets/flask to 10 mm, 44/flask to 7.5 mm, 176/flask to 5.0 mm, 704/flask to 3.5 mm; shake flask; *Aspergillus nidulans (72)*

Table 3
Effects of Media Composition on Pelleted vs Filamentous Growth

Media composition	Impact	Specific example
Variation of carbon source present	Dispersed mycelia and clumps	Presence of lactose, lactic acid, or peanut oil; 1.5×10^6 spores/mL inoculum; 0.5-L fermentation; *P. chrysogenum (73)*
	Highly branched and semipelleted	Presence of glucose (0–10 g/L) and molasses (0–15 g/L) at various concentrations and combinations, 1.5×10^6 spores/mL inoculum, 0.5-L fermentation, *P. chrysogenum (73)*
Complex nitrogen source present	Decrease in pellet size, larger number of filaments attached to pellet	Large pellets with use of Pharmamedia and smaller pellets with larger number of filaments attached with use of gluten meal, shake flask, *Streptomyces fradiae (74)*
Switch from complex to defined nitrogen source	Change from pulplike to pelleted growth	Corn steep liquor (pulplike) and ammonium sulfate (pellet), *P. chrysogenum (75)*
Addition of yeast extract to minimal media	Change from filamentous to pelleted form	When yeast extract added in range of 0.5–1.5 g/L, change from filamentous to pelleted morphology; as yeast extract increased further from 2 to 7 g/L, increase in pellet size (2–4.7 mm) and decrease in density (0.09–0.04 mg/mm^3); 1×10^7 spores/mL inoculum; 1-L fermentation, *A. niger (76)*
Increase in nitrogen (ammonia) concentration	Switch from loose and fluffy pellets to compact and smooth pellets	100–500 mmol/L NH_4NO_3: compact; 1–5 mmol/L NH_4NO_3: loose; shake flask; *Myrothecium verrucaria (77)*
Greater additions of ammonium ion	Larger pellet size, fewer filaments	Larger pellets (200–400 μ) with small amount of filaments with use of 200 m*M* ammonium ion, shake flask, *S. fradiae (78)*
	Smaller pellet size, more entangled filaments	Smaller pellets (50–200 μ) with large entangled filaments with use of 20 g/L amino acid mixture (10 g/L aspartic acid and 10 g/L glutamic acid), shake flask, *S. fradiae (78)*
Greater calcium concentration	Loose pelleted growth favored, shorter and more highly branched mycelia	Loose pelleted growth with carbon source diverted into citric acid production when 0.5 g/L calcium chloride used, more branched growth/pellets with carbon source used more for biomass when 0 g/L calcium chloride used, 0.75-L fermentation, 10^5 spores/mL inoculum, *A. niger (79)*

Greater monopotassium phosphate concentration	Increased mycelial growth	With increase in monopotassium phosphate concentration above 0.02 wt%, increase in mycelial growth and thus viscosity; 4-L fermentation; 0.5–2.5 $\times 10^4$ spores/mL inoculum, *A. niger (31)*
Increased intensity of sterilization cycle	Change in morphology from pelleted without sporulation to loose growth followed by sporulation	1×10^5 spores/mL inoculum, 2600-L fermentation, *A. niger (80)*
Addition of acetate	Increase in pellet fluffiness and decrease in pellet size	0.2 wt% ammonium acetate, shake flask, *A. niger* strain R-8 *(39)*
Addition of fatty acids such as oleic acid	Decrease in pellet density, increase in pellet diameter	0.1–0.3 wt% oleic acid; mean diameters of 0.35 mm for control, 0.40 mm for 0.1%, 0.44 mm for 0.3%; accelerated enzyme production; 20-L fermentation; *Montierella vinacea (81)*

Table 4
Effects of Media Additives on Pelleted vs Mycelial Growth

Media additive[a]	Impact	Specific example
Ferrocyanide	Decrease in pellet size and increase in roundness	Above 10 ppm, growth of pellets from 0.2–0.5 to 1.2–2.5 mm after 5 d; below 10 ppm, larger (up to 6 mm) and irregularly shaped pellets; pellets hard in both cases; with 0 ppm, formation of soft filamentous pellets of varying size and shape; 2.5-L fermentation; 200 pellets/mL inoculum; *A. niger (82)*
Metal-complexing agents	Small discrete pellets with smooth exterior	1.04 m*M* EDTA added to medium containing 1.04 m*M* metal ions (Fe, Cu, Zn, Mg), shake flask, 2×10^6 spores/mL, *A. niger (83,84)*
Polymer additives (nonionic PEG20,000 and anionic CMC)	Smaller pellets, eventually filamentous growth, reduction in spore agglomeration, increase in broth viscosity (lower oxygen solubility)	PEG20,000: filamentous growth with 20 w/v%, pelleted growth with 0–10 w/v%; CMC: filamentous growth with 2.0 w/v%, pellets formed with 0–1.5 w/v%; different optima for product titer; no exact diameters given; shake flask; 1.3×10^6 spores/L inoculum; *P. chrysogenum (85)*
Sodium alginate and dextran	Increase in viscosity, switch from pellet to pulplike growth	Increase from 1 to 40 cp may have affected spore agglutination as well as viscosity, shake flask, *A. niger (21)*
Anionic polymer, (Carbomer [Carbopol] 934, B.F. Goodrich)	Change from pellets to dispersed mycelium	Prevention of agglomeration of spores into clusters with 0.5 kg/m^3 Carbopol; reduction of spore/pellet ratio by 30-fold, reduction of formation of wall growth, and production of filamentous growth with 3 kg/m^3 Carbopol; opposite effect with cationic polymers; shake flask; 10^{10} spores/m^3 inoculum; *A. niger (12)*
Anionic polymer (crosslinked polyacrylic acid, water-soluble acrylic resins)	Reduction in aggregation of spores and hyphae and in pelleting	2 g/L Junlon 111 or 1.25 g/L Junlon 110, $4–8 \times 10^5$ conidia/mL, shake flask, *A. niger (86)*
Anionic polymer (polyacrylic acid)	Reduction in flocculation of spores	For 0.3 w/v%, "pulpy" 1-mm structures vs 4- to 8-mm "hard-packed" pellets for 0 w/v%, 1.5-L fermentation, *A. niger (22)*
Nonionic surface-active agents	Change from pellets to homogeneous (pulp) and filamentous form	0.05% sucrose monostearate or Span 20, shake flask, 10^5 spores/mL inoculum, *A. niger (21)*
Nonionic surface-active agent (polysorbate [Tween]) 80)	Change from mycelia to large "globose" pellets	0.05% Tween-80, shake flask, 10^5 spores/mL inoculum, *A. niger (21)*
Nonionic polymer (PVP)	Change from pellets to disperse filamentous growth	1–2 w/v% PVP, shake flask, 10^5 conidia/mL inoculum, *A. niger (20)*

[a]PEG, polyethylene glycol; CMC, carboxymethylcellulose; PVP, polyvinylpyrrolidone.

Table 5
Effect of Fermentation Parameters on Pelleted vs Mycelial Growth

Fermentation parameter	Impact	Specific example[a]
Decrease in specific growth rate	Lower clump area (and thus lower estimated diameter)	Growth rate of 0.09 h^{-1}: 265 µ; 0.075 h^{-1}: 250 µ; 0.05 h^{-1}: 150 µ; 7-L chemostat; *A. niger (87)*
	Shorter pellet core diameter	Growth rate of 0.10 h^{-1}: 925 µ; 0.08 h^{-1}: 860 µ; 0.06 h^{-1}: 775 µ; 10-L chemostat; *A. niger (45)*
Increase in pH	Larger pellets	For broth pH of 4.5, 700-µ pellets; for broth pH of 2.1, 400-µ pellets; 8-L fermentation; *A. niger (88)*
	No morphologic effect	5.5 < pH < 7.0, 9- to 10-L fermentor, *P. chrysogenum (89)*
Decrease in pH	Filamentous growth	Critical pH of 2.3 for filamentous growth; pellets formed in more alkaline conditions up to pH 4.5; investigation using defined medium identical dcw and glucose/ammonium uptake regardless of morphology, 1.07×10^5 spores/mL inoculum, *A. niger (90)*
Decrease in DO	No morphologic change	12- to 300-mmHg range examined, 9- to 10-L fermentation, *P. chrysogenum (89)*
Increase in CO_2 in feed air	Abnormal morphological changes	Highly branched tips and swollen branch points, 12.5-L fermentation, *P. chrysogenum (91)*
	Longer mean hyphal and branch length	0–18% CO_2, continuous culture, 8000-L fermentor, *A. niger* A60 *(92)*
Decrease in dissolved CO_2 level	Slower germination of spores	99 ± 21 vs 44 ± 14 h, 9- to 10-L fermentation, *P. chrysogenum (89)*

[a]dcw, dry cell weight (g/L).

The broth pH effect on pelleting vs filamentous growth may be owing to alteration of spore surface properties responsible for coagulation *(6)*. Although higher agitation rates can prevent pellet formation for strains with coagulative pellet types, the effect is not as clear for strains with non-coagulative pellet types *(6)*. In general, filamentous growth is dominant at higher agitation rates; compact pellets are observed at lower rates, with looser pellets observed at the lowest rates *(23,24)*. The lack of any particular nutrient (including oxygen) may lead to pellet formation, but there are counterexamples to this relationship *(16)*.

Pellet Diameter

Pellet diameter is critical to evaluating the acceptability of pellets in a particular process. Pellets that are too large form a necrotic core owing to insufficient oxygen transfer rates relative to oxygen usage (diffusion in the presence of a reaction). For example, larger pelleted growth (1–3 mm in diameter) reduced the extent of secondary metabolite accumulation for penicillin production by *Aspergillus nidulans*; smaller micropellets (0.1–0.4 mm in diameter) performed between larger pellets and filamentous growth. This behavior was the result of oxygen limitation within larger pellets, a hypothesis confirmed by inducing oxygen limitation in a filamentous culture *(25)*.

In many cases, the physical meaning of the pellet diameter is not straightforward. It can refer to the diameter of the entire pellet clump or just the core portion (not counting loose annular material). Furthermore, in some instances, clumps are irregularly shaped so researchers have elected to report pellet perimeter values. In other instances, researchers have measured pellet cross-sectional areas. For this article, all pellet sizes (areas or circumferences) reported by various researchers were converted to estimated effective average diameters for ease of data comparison.

Effect of Pellets on Productivity

The effect of pellets on productivity is specific to the process under investigation. In some cases, immobilization in pellet form shifts fungal metabolic activities toward secondary metabolism *(16)*. The pellet form can lead to higher productivity when broth viscosity becomes lower, which, in turn, improves aeration efficiency and mixing *(26–28)*. However, there are several counterexamples for which morphology does not affect productivity and thus can be manipulated independently *(29,30)*.

Application to *Aspergillus* Cultivation and Citric Acid Production

Citric acid production by *A. niger* is a major example for which pelleted growth has been widely utilized. There are several notable articles on the growth of *A. niger* and citric acid production *(25,31–43)*, on either constitutive or heterologous enzyme production *(44,45)*, and on organic acid production *(46)*. Specifically, three articles give insightful overviews of the state of citric acid production at the time they were written *(47–49)*.

In general, citric acid cultivations use a defined medium containing sugar (typically sucrose but often glucose), potassium phosphate, magnesium sulfate, inorganic nitrogen (such as ammonium salts of carbonate, sulfate, chloride, or nitrate), and trace elements (such as zinc, manganese, ferrous or cupric sulfate, manganese chloride, and/or sodium molybdate). Quite often, no trace elements are required despite the use of defined medium.

Cultivation conditions can influence pellet formation (as well as citric acid productivity), so they are listed in Tables 6–9 when available for the cited investigation. These conditions also vary considerably among the different literature reports. Spore inoculum is used in a concentration range of 10^3–10^6 spores/mL (based on inoculated production broth volume); cultivation pH ranged from 2.0 to 6.5; cultivation temperature ranged from 24 to 34°C; and production vessel volume ranged from shake flasks to 50-L fermentors, with one study at 2600 L.

Several key points from studies of citric acid production were applied to the process development of *Aspergillus* and other fungal strains as well as one *Streptomyces* culture.

Variation in Inoculum and Production Temperatures

Although practically no citric acid is produced at 37°C in various *A. niger* strains, high yields of citric acid are produced between 24 and 30°C despite the fact that cell mass is as high at 37°C as it is at 24–30°C. The decline in citric acid yield may be a result of the higher citrate metabolism at 37°C so that it does not accumulate *(47)*. This lead was applied to the production of indole diterpenes by *A. niger* to attempt to minimize undesirable formation of organic acid.

Maintenance of Low pH Set Point at 4.0 or Above

The pH of citric acid broths sometimes has been maintained above 4.0 to prevent damage to 316-L stainless steel fermentors (solubilization of metals such as manganese and iron) over the course of a 28-d cultivation of *A. niger* (Tables 6–9). This restriction caused difficulty in reproducing laboratory shake-flask indole diterpene titers in which pH was not controlled and typically declined to 1.0 to 2.0.

Variation in Agitation Rate

For citric acid fermentations, the critical DO level for product formation typically is significantly higher than for culture growth *(37)*. Higher agitation rates were explored for several model cultures to maintain DO above 50–80% sat (calibrated to air at ambient pressure) for process development.

Role of Morphology

In the citric acid process, a spore inoculum is commonly used that first forms hyphal agglomerates followed by spherical pellets. Optimal citric

Table 6
General Influences on Citric Acid Production

Item	Impact	Specific example
Source of inorganic nitrogen	Reduced slime-forming tendencies (lowered viscosity), which improved aeration efficiency	Performance of ammonium carbonate (mixture of ammonium biocarbonate and ammonium carbamate) better than ammonium sulfate and other nitrates, 5–25 × 10^6 spores/L, 4-L fermentation, 24–34°C, pH 2.5, *A. niger (31)*
Pelleted inoculum	Pellet growth phase	In general, for citric acid production, inoculum of pellets of 0.2–0.5 mm (diameter of "loose" form), causes production rise in pellet concentration to 1.2–3.6 × $10^8/m^3$ of 1 to 2 mm in diameter; by end of fermentation pellets much smoother; tip speed of 0.6 m/s typical; pH 2.0–3.0, *A. niger (6)*
Production culture pelleting	Decrease in citric acid production when culture was not pelleted	2600-L fermentation, 1 × 10^6 spores/L, 29–30°C, pH 5.6, *A. niger (80)*
Typical production conditions	Kinetic relationship between growth and citric acid production	0.5 vvm aeration, 1650 rpm in 50-L pilot fermentor, 5 × 10^7 spores/L, 30°C, pH 3.0, *A. niger (35,38)*

Table 7
Effects of Media on Citric Acid Production

Item	Impact	Specific example
Culture grown on glucose in defined medium	Accumulation of polyols (glycerol, arabitol, erythritol, mannitol), which are reconsumed	More oxygen than sugar required for catabolism of polyols later in fermentation (RQ decreases), 50-L fermentor, 30°C, pH 3.0, 5×10^4 spores/mL, *A. niger (40)*
Addition of Pharmamedia (cottonseed flour) to defined medium	High levels of clumps and aggregation (>87%)	Pharmamedia suspended, boiled in water for 10 min, then filtered to remove solids before adding to medium; chemostat; 7-L fermentation; 28°C; pH 4.5; *A. niger (87)*
Addition of ground nut oil to defined medium	Increase in citric acid production	Oil rich in acetyl coenzyme A, which acts as precursor of citric acid cycle; pH 4.5–5.0; 29–30°C; shake flask; *A. niger (93)*
Addition of 500–1000 ppm (0.05–0.1 wt%) morpholine to defined medium	Faster citric acid production rate and stabilizing effect on fungus	Pellet diameter <0.5 mm and on average >0.1 mm with gross granular appearance, role of morpholine not clear, $50–100 \times 10^6$ spores/L, 4-L fermentation, pH 2.5, 30–32°C, *A. niger (80)*
Nitrogen source growth limiting in defined medium	After depletion of nitrogen, beginning of citric acid production	Lower intracellular ammonium levels, deficiency of Mn leading to citric acid production, direct correlation of specific OUR with intracellular ammonium ion concentration, yield maximized by pulse feeding of ammonium, 3-L fermentation, pH 3.8, 30°C, 10^8 spores/L inoculum, *A. niger (43)*

Table 8
Effects of Nature and Concentration of Trace Elements on Citric Acid Production

Item	Impact	Specific example
Addition of 100 mg Cu^{+2}/L, 50 mg Fe^{+3}/L, or 0.1 mg Mn^{+2}/L into defined medium	Growth same regardless of addition; with Mn^{+2} least amount of citric acid accumulated, followed by Fe^{+3}; Cu^{+2} similar to control	Unclear reason for effect but seemed to prove it was not owing to inhibition of aconitase activity (aconitase is second enzyme of citric acid cycle that catalyzes breakdown of citrate within cycle), 50-L fermentor, pH 3.0, 30°C, 5×10^4 spores/mL inoculum, *A. niger (36)*
Presence of manganese (100 ppb $MnCl_2 \cdot 4H_2O$) in defined medium	Increase in cell growth, decrease in sugar consumption, drastic decrease in acidogenesis	Shift in fungal metabolism toward biosynthetic side reactions owing to inorganic nutrient limitation in presence of manganese, shake flasks, pH 3.0, 28°C, 5×10^5 spores/mL inoculum, *A. niger (34)*
Increased Fe^{+2} concentration (varied from 0 to 3 mg/L) with sucrose-based medium	Pellet sizes largest and fall in citric acid production as Fe^{+2} concentration increased to 1.5 mg/L Fe^{+2}	Effect possibly owing to oxygen transport rather than Fe^{+2} presence, 4-L fermentor, *A. niger (94)*

Table 9
Effects of Fermentation Conditions on Citric Acid Production

Item	Impact	Specific example
Increase in agitation rate	Decrease in broth pH, residual sugar, citric acid yield/concentration; increase in biomass	Decline in broth pH to lower value of 0.2 units per 100-rpm increase; increase in biomass about 1 g/L per 100-rpm increase; decrease in residual sugars; decrease in citric acid yield on sugar of 4 wt% per 100-rpm increase; decrease in citric acid of 7–14 wt% per 100-rpm increase; above 300 rpm, formation of 2- to 3-mm pellets within 24–48 h, which were later crushed into homogenous mycelial dispersion; 12-L fermentor; 2.7×10^5 spores/mL inoculum; pH 6.5; 30°C; *A. niger (41)*
	Highest productivity when agitation rate favored pellet formation	At 91 rpm formed pellets, at 155 rpm grew in filamentous form, at 68 rpm insufficient oxygen transfer; 400-L fermentor; pH 5.0; 30°C; 1×10^8 spores/L inoculum; *A. niger (64)*
Counterflow mixing system	Higher citric acid concentration, sugar consumption rate, and citric acid yields on sugar and biomass	3.0-L fermentation, pH 5.5, 32°C, *A. niger (95)* 5-L fermentation, pH 1.7–2.0, 32°C, 3.5×10^6 spores/mL inoculum, *A. niger (14)*
Interruption of aeration (20 min)	Loss of ability to produce citric acid, increase in airflow rate and growth rate when reinstated	50-L fermentor, pH 3.0, 30°C, 5×10^4 spores/mL inoculum, *A. niger (37)*
Higher DO level	Higher biomass, CER, and OUR	With use of 60, 80, and 145 mbar of DO, biomass of 20, 50, 80 g/L; CER of 55, 55, 65 mmol/L·h; OUR of 75, 90, 105 mmol/(L·h); and RQ of 0.73, 0.61, 0.62 (calculated); 50-L fermentor; pH 3.0; 30°C, 5×10^4 spores/mL; *A. niger (37)*
Midcycle control of pH	Lower cell mass specific protease activity	pH control at 4.0 vs pH rise at latter fermentation stages for uncontrolled case, 10-L fermentor, pH 4.0, 25°C, *A. niger (96)*

DO, dissolved oxygen; CER, carbon dioxide evolution rates; OUR, oxygen uptake rate, mmol/(L·h); RQ, respiratory quotient.

acid production is achieved when the pellet growth form dominates and broth is pseudoplastic *(27)*. By contrast, some researchers have observed that if the mycelial form is made to dominate using media additives, high specific productivities of citric acid can be obtained *(50)*. Consequently, even with a process, as well studied as citric acid, the role of morphology is not always clearly delineated.

The use of additives, early exponential phase transfer timing of inoculum shake flasks, and variations in agitation rate to change seed and/or production broth morphology were explored for early phase process development for five model fungal and filamentous bacterial cultures. Only qualitative, indirect, and macroscopic assessments of the morphologic effects of these changes were conducted; no direct viscosity testing or microscopic measurements were undertaken.

Materials and Methods

General

Laboratory seed was cultivated in two stages: a 250-mL and a 2-L shake flask. Nonbaffled Erlenmeyer seed flasks were sterilized at 122°C for 30 min (first stage) or 40 min (second stage), inoculated, then shaken at 220 rpm (with a 2 in throw or stroke) in an incubator room. Fermentor sterilizations were conducted at 122–124°C for 40 min for seed fermentors and 45 min for production fermentors. Deionized water was used for all stages of cultivation and all nutrient solutions. Poststerilization cerelose (glucose monohydrate) additions used a 50 wt% solution. Broth pH was controlled with 25 wt% NaOH (caustic) and/or 25 wt% H_2SO_4. DO was calibrated to 100%sat in air at ambient pressure. Off-gas was measured using either a Perkin-Elmer MGA1200 *(51)* or a VG Prima 600S *(52)* mass spectrometer system.

Depending on the process, first-stage seed flasks, typically inoculated with 1 to 2 mL of seed from a frozen vial into 50 mL of medium (2–4 vol%), were cultivated for 1–3 d, and second-stage seed flasks, inoculated with 10 mL of seed from the first-stage seed flask into 300–500 mL of medium (2.0–3.3 vol%), were grown for 1 to 2 d. First-stage seed fermentors were inoculated with 1.5–3.5 L of either laboratory or frozen bag seed *(53)* into 180 L of medium (0.83–1.9 vol%) and were cultivated for 1 to 2 d. Finally, for most cultivations, 10–25 L of seed was transferred to about 600 L of production medium (1.6–4.2 vol%).

Seed-vial preparation conditions were reasonably similar to seed inoculum flask conditions. Both sets of conditions were developed to minimize pellet formation, reduce growth lags, and establish reasonable per-stage seed transfer times. Control of seed pelleting was critical to influencing production-scale morphology and ensuring success of early phase fermentation scale-up. Details of seed and production media as well as fermentation conditions are given in the appendix for the five model cultivations.

A. niger

A strain of *A. niger* (MF6670) was cultivated at the 600-L pilot scale to produce indole diterpene compounds (mol wt of about 400) to treat indications of atherosclerosis. Large-volume frozen bagged seed *(53)* was prepared from pilot-scale seed fermentors to reduce repetitive, less convenient, and sometimes variable shake-flask seed train development. (Inoculum flask transfer times were <16 h for each of the two shake-flask seed stages.) Seed flasks were transferred promptly during the early exponential growth stage to minimize pellet size to about 0.5 cm or less.

Seed-flask medium was KF *(54)* and seed fermentor medium was YME. Seed fermentors were inoculated with either laboratory or frozen bagged seed. DO declined from 170 to 50–60%sat. Broth pH began at 6.3 and declined to 4.0 before the oxygen uptake rate (OUR) reached its transfer target of 5 to 6 mmol/(L·h); caustic was used to control pH at 4.0 prior to inoculation. Production fermentor medium was JJCB without trisodium citrate. At 22°C, DO declined from 170 to about 90%sat at 18–20 h, then rose steadily for the remainder of the fermentation, and DO control was not needed. At 28 and 35°C, the DO dropped to 50%sat and then was cascaded with agitation rate, consistent with higher OUR. Broth pH began at 5.5 and declined to 4.0, at which time the pH was controlled. Owing to the presumed high production rate of organic acid, these cultivations utilized an unusually large amount of caustic of about 5 gal (20 L)/d for 600 L of broth (3.3 vol% addition/d).

A. unguis

A strain of *A. unguis* was cultivated at the 600-L pilot scale to produce unguinol (mol wt of about 450), a bile salt hydrolase inhibitor with potential as a poultry growth promoter *(55)*. Seed-flask medium was YMEJ, YME with cerelose sterilized together supplemented with 1.5 g/L of Junlon (a polyacrylate, Nikon, Juyaki, Osaka, Japan, PW-110), which was not found necessary in seed or production fermentors. Despite the addition of Junlon, which reduced pelleting severity (the addition of 2 g/L of agar or separate cerelose sterilization had little effect), the culture still pelleted considerably, making it challenging to pump into seed tanks through 3/8-in. od (3/16-in. id) silicone tubing (Masterflex size 15; Cole Parmer, Vernon Hills, IL). Because pellets were soft and compressible, manual massaging of the transfer line during pumping prevented clogging. Large-volume frozen bagged seed *(53)* also was prepared, which minimized transfer problems, because pellet sizes were reduced significantly in agitated seed fermentors.

Seed fermentor medium was YME. DO decreased from 170 to 120%sat. Broth pH began at 6.1 and declined to 5.1 when the OUR reached 5 to 6 mmol/(L·h). Production medium was JJCB. DO decreased from 170 to 50%sat at 40 h and then was controlled. Broth pH was controlled between 4.5 and 7.5.

S. araneosa

S. araneosa (ATCC 36386) was cultivated at the 600- and 15,000-L pilot scales to produce sordarin (mol wt of about 500), an antifungal that selectively inhibits fungal protein synthesis by impairing the function of the eukaryotic elongation factor, EF2 *(56,57)*. Similarly to *A. unguis*, after initial challenges with laboratory shake-flask inoculum preparation, large-volume frozen bagged seed *(53)* was prepared from agitated seed fermentors to minimize pellet sizes observed in second-stage seed shake flasks and used to inoculate seed fermentors. Seed-flask medium was KF medium without agar *(54)*.

Seed-tank medium was AD-2 with 28 g/L of cerelose added poststerilization. Scale-up of the laboratory shake-flask medium was undesirable owing to the presence of tomato paste and oat flour components that were prone to foaming. Because scale-up could not be delayed until a suitable fermentor seed medium was developed, this seed medium actually was modified production medium. DO declined from 170 to 100%sat and initial broth pH declined from 7.4 to 7.0 at 30 h, with OUR reaching 2.3–2.5 mmol/(L·h) at the time of transfer.

Production medium (AD-2) was the same composition as seed medium except 166 g/L of cerelose was added poststerilization. At the 600-L scale, agitation reached about 220 rpm at its peak. Broth pH remained in the range of 5.8–7.4 without control. The 15,000-L scale cultivation was inoculated with two seed-tank fermentors (315 L, 3.15 vol%). DO was controlled by manually raising agitation from 60 to 110 rpm and airflow from 5000 to 9000 L/min to maintain DO above 40%sat. NaOH was added manually to maintain broth pH above 5.3 because the size of automatic shots caused broth pH to rise dramatically from 5.3 to 6.3.

S. roseoflavus

A mutant culture of *S. roseoflavus* was cultivated at the 600-L pilot scale to produce a novel cyclic peptide (mol wt of about 1400) with potential antibiotic potency against methicillin-resistant *Staphylococcus aureus*. Seed-flask culture (grown in ISM-3 medium) was inoculated into ISM-3 seed fermentor medium. Broth pH began at 6.4, then declined, but not below 4.8–5.0 by the time the OUR reached its target value of 7, 14, or 19 mmol/(L·h). There were no seed morphology issues associated with this process.

Production fermentors contained FR23 medium. After production medium was transferred to fermentors and awaited sterilization, DO declined to zero nearly immediately, indicating a higher presterilization bioburden than normally observed. During the sterilization heat up (i.e., prior to 80°C), steam at 30 psig was blown up the fermentor bottom valve for 30 s to suspend any solids that might have collected in the deadleg between the tank and its bottom valve (which was not flush mounted). When the medium reached 80°C, the batch was held at this temperature for 5–15 min to permit spores to germinate and to ensure starch solubilization. During

this heat-up phase (80–95°C), several (about 24) inches of foam developed, which decreased substantially when tank internal steam sources (both above and below the liquid surface) were opened, then reappeared when internal steam sources were closed. This behavior was most likely influenced by changes in media surface tension with temperature as a result of the soluble starch and/or Pharmamedia. Owing to substantial fermentor headspace, this foam did not reach the fermentor vent line piping.

Broth pH was controlled between 5.3 and 5.5, with care taken not to permit it to rise above 7.0, to maximize product stability. There was some coating of the pH probe by the broth during the fermentation cycle, so grab samples were analyzed off-line to compare to fermentor pH probe readings. Despite high airflow and agitation rates, midcycle foaming was not apparent, perhaps because fermentor back pressure also was increased midcycle to 1.2–1.5 kg/cm^2 to raise DO levels above 50%sat.

C. affinis

C. affinis was cultivated at the 600-L pilot scale to produce micafungin, an antifungal precursor that is a 1,3 β-D-glucan synthetase inhibitor *(58)*. Seed vials were prepared using KF medium *(54)* with 4 g/L of agar to minimize pelleting, which initially was severe enough to prevent pipeting of broth into vials. Seed-flask culture (grown in LYCP-5 medium) was inoculated into LYCP-5 seed fermentor medium. Broth pH began at 5.6 and remained largely unchanged by the time the OUR reached its target transfer value of 3–6 mmol/(L·h).

Production medium was BB1. Care was taken to ensure that airflow rates were sufficiently high to reduce power draw for the higher agitation rates. Broth pH was controlled between 4.5 and 6.2.

Cultivation Results

Details of the pilot plant scale-up and early process development challenges are described next for the five model cultivations and are summarized in Tables 10 and 11. These production batches utilized seed trains that minimized the impact of pellet formation so that scale-up was focussed on pilot-scale processing issues. On-line and off-line data were used to identify expected and unexpected aspects of cultivation performance.

A. niger

The growth of *A. niger* was studied at the 600-L pilot scale under various conditions (temperature of 22–35°C, initial agitation rates of 100–350 rpm, initial airflow rates of 0.15–0.5 vvm) using a semidefined medium that was operationally easier than the original laboratory-scale medium containing oat flour and tomato paste.

At 22°C, the OUR rose to 9–12 mmol/(L·h) after about 50 h, then declined to about 2 mmol/(L·h) by 250 h. Peak OURs were 9.0 mmol/(L·h) at 30 h at 28°C and 11 mmol/(L·h) at 12 h at 35°C, demonstrating faster

Table 10
Comparison of Media (and Key Ingredients), On-Line Monitoring, and Titer Reproducibility for Five Model Cultivations[a]

Cultivation	Seed shake-flask medium	Seed fermentor medium	Production fermentor medium	On-line monitoring characteristics/ implications	Fermentation titer reproducibility (% rsd)
A. niger	KF (corn steep powder, tomato paste, oat flour, glucose)	YME (yeast extract, malt extract, cerelose)	JJCB (yeast extract, malt extract, ammonium sulfate, mannitol, cerelose)	Low midcycle RQ of 0.2–0.3/broth CO_2 accumulation High base pH adjustment activity/ organic acid production	N/A
A. unguis	YMEJ (YME with polyacrylate)	YME	JJCB (with trisodium citrate)	Midcycle DO and pH rise/ nutrient depletion Power draw/ relative broth viscosity Drop in OUR and CER/ glycerol exhaustion	21.0 (Rushton impellers)
S. araneosa	KF	AD-2 (modified production medium)	AD-2 (yeast extract, glycerol, monosodium glutamate)	Steady RQ of 1.0/ glucose metabolism Shifts in midcycle pH/ shifts in metabolism	9.0 (Maxflo T impellers) 20.9 (different impellers)
S. roseoflavus	ISM-3	ISM-3 (yeast extract, malt extract, ferric chloride, cerelose)	FR23 (yeast extract, cottonseed flour, soluble starch, sucrose, cerelose)	Presterilization DO decline to 0 %sat/ presterilization bioburden	18.8 (different impellers)
C. affinis	LYCP-5	LYCP-5 (yeast extract, cottonseed flour, cerelose)	BB1 (yeast extract, cottonseed flour, glutamic acid, mannitol)	Shifts in midcycle pH and RQ/ shifts in metabolism Power draw/ relative broth viscosity	36.0 (different impellers)

[a]All fermentations utilized target oxygen uptake-rate values to transfer inoculum from seed to production fermentors. Impellers are described elsewhere *(60)*. N/A, not available; DO, dissolved oxygen; RQ, respiratory quotient; CER, carbon dioxide evolution rates; OUR, oxygen uptake rate; KF, seed shake flask medium; YME, seed shake flask and seed fermentor medium; JJCB, production fermentor medium; LYCP-S, seed shake flask and seed fermentor medium; ISM-3, seed shake flask and seed fermentor medium; AD-2, production fermentor medium; BBI, production fermentor medium; FR23, production fermentor medium.

Table 11
Comparison of Initial Process Scale-Up Issues and Their Resolution for Five Model Cultivations

Cultivation	Seed issues/resolution	Production issues/resolution	Achievement of laboratory-scale titer	Comments
A. niger	Fast shake-flask growth and pelleting/ early exponential phase transfer to next stage, frozen bagged seed from agitated fermentor	"Caking" causing airflow and pressure control problems; expensive mannitol substrate/manual control valve stroking, steaming sparger line, higher agitation and airflow rates; cheaper cerelose substituted	No	Development of scalable production medium unsuccessful
A. unguis	Pelleting/Junlon addition to shake-flask medium, frozen bagged seed from agitated fermentor	Complex production medium foaming, product instability/semidefined medium development, higher calcium carbonate with postbatch acid-cleaning solution	Yes (exceeded)	Development of scalable production medium successful
S. araneosa	Pelleting, operationally difficult seed medium/ frozen bagged seed, modified production medium used for seed fermentor stage	pH excursions, postbatch cleanability/manual pH addition, lower calcium carbonate	Yes (exceeded)	Successful scale-up to 15,000-L scale based on one prior 600-L run
S. roseoflavus	None	Erroneous readings owing to pH probe coating, agitator overload owing to broth viscosity, foaming during sterilization/ manual pH check using off-line sample, higher seed fermentor OURs at transfer and higher production fermentor airflow rates, lower batching volumes	Yes	Successful initial scale-up
C. affinis	Pelleting/agar addition to shake flasks	Agitator overload owing to broth viscosity/midcycle sterile water additions and decrease in medium concentration of complex nitrogen source	Yes	Successful initial scale-up

OURs, oxygen uptake rates

growth at higher temperatures. At 28°C and 350-rpm initial agitation rate (vs 200 rpm) using a Rushton impeller, peak OUR reached only 6.0 mmol/(L·h) at 30 h, indicating some culture sensitivity to shear. When 140 g/L of cerelose was substituted for the 70 g/L of mannitol (with 20 g/L of additional cerelose present in both cultivations), OURs at 28°C rose to 12–14 mmol/(L·h), indicating adequate growth on this cheaper carbon substrate.

At 28°C, the respiratory quotient, RQ, rose to about 1.0 by 12–15 h; declined to a plateau of 0.65 from 15 to 25 h; declined further to a plateau of 0.2–0.3 by 50 h; and then rose slowly, reaching 0.4–0.6 by 240 h. This unusually low RQ was not caused by a mass spectrometer measurement problem. Potentially high media solubility of CO_2, owing to high citric acid byproduct concentrations, may have reduced CO_2 evolution rates (CERs) *(59)*. Midcycle RQs were still low at 0.2–0.3 (rising to 0.4–0.6 by 240 h) even when all the mannitol was replaced by cerelose in the medium. At 22°C, similar RQ values and patterns were observed.

At 22°C, cell solids rose to 33–38 wt% at 90–100 h, dipped to 25–28 wt% at 150–200 h, then rose to 30–40 wt% by 300 h. When temperature was increased to 25°C, cell solids rose to only 22–25 wt% by 80–100 h, dipping to 15–18 wt% at about 130–170 h, and then rising to 22–25 wt% by about 200 h. Thus, as temperature increased, growth patterns were similar although slightly faster, and peak cell densities were lower.

At 22°C, glucose was utilized initially at a rate of 0.78 ± 0.10 g/(L·h) and was exhausted by 90 h. Mannitol was used initially (concurrently with glucose), with about 20 g/L remaining after 400 h. At 28°C, glucose was exhausted by 22 h, suggesting increased metabolic rates as temperature increased despite lower cell mass. When 140 g/L of cerelose replaced the 70 g/L of mannitol at 28°C, glucose was exhausted by 45 h, suggesting that the presence of mannitol slowed glucose utilization.

Fermentations were difficult to run to completion because of midcycle (250–300 vs 600 h) problems controlling airflow and back pressure, most likely owing to "caking" around the sparger outlet and/or back pressure control valve, despite the fact that no vent filters were being utilized. This condition caused airflow rates to decline and back pressures to increase gradually over time, although it was not clear whether airflow rate or back pressure behavior was the cause or effect. Manual stroking of the back pressure control valve and direct steaming through the sparger line for 2–5 min often seemed to fix the problem. Postbatch examination of the 0.2-μ sparger air filters indicated some slight discoloration, but when these filters were used in a subsequent test batch, performance was acceptable. Vendor analysis of the filters did not reveal anything unusual. Because no other process scaled up to date in the pilot plant exhibited this unusual behavior, it was concluded that this was a process-specific problem.

Despite attempts to vary culture conditions (i.e., initial agitation, impeller type and airflow rates, and temperature) to optimize morphology and minimize organic acid byproduct formation, scale-up of this culture

was not successful, with essentially none of the desired product produced after 25–28 d. Additional efforts were needed to translate the laboratory-scale fermentor medium (containing oat flour and tomato paste) and pH control strategy to pilot-scale conditions, but these efforts were curtailed owing to changing development priorities.

A. unguis

Initial cultivations first were scaled up using a complex production medium containing tomato paste and soybean meal but using YME for the seed flasks and fermentors. Production fermentors foamed considerably during sterilization, and several batches (approximately three of six) became contaminated midcycle. This high contamination rate was presumed to have resulted from inadequate wetting and sterilization of solid components, potentially owing to their entrapment in the foam layer and/or deposition on tank internals after foam subsided. Production fermentor OURs rose to 16 to 17 mmol/(L·h) by 30 h, remained steady up to 170 h, and then slowly declined until harvest. Initially, broth pH declined from 6.2 to 4.8, then rose to 6.4 at 30 h, corresponding to when acid byproducts of carbon metabolism were overcome by basic byproducts of nitrogen source metabolism. Titers at 300 h ranged from 0.6 to 0.9 mg/L (Table 12).

Production medium then was switched to JJCB medium. No foaming was observed during sterilization with this new semidefined medium. As in complex medium fermentations, OUR rose to 12.5 mmol/(L·h) by 45 h, then declined linearly until harvest at 400 h. Glucose declined to zero sometime between 40 and 60 h. In addition, RQ dropped from 0.9 to 0.7 at 45 h, indicating a switch from cerelose to glycerol utilization. Glycerol then declined gradually and became exhausted by 180–210 h. Peak titer was reached shortly afterward. Similarly to complex medium fermentations, broth pH began at 6.2, declined to 5.3 at 45 h, rose to 6.2 as RQ decreased, and then remained fairly constant. Ammonia declined from 38–45 mmol/L to 0 by 90 h and production began at 100 h. Phosphorus declined steadily from 100–110 to 60 to 80 mg/L by 40 h, indicating no evidence of phosphate regulation. Interestingly, the unusually high calcium carbonate medium concentration was critical to titer stability in the broth. Titers reached 1.0–1.2 g/L at 214 h, with a faster overall production rate than that observed at the 20-L laboratory scale (Table 12, run 1) and slightly higher titers than were observed with the initial complex production medium (Table 12).

Despite unusually high titers for an early phase project, pilot-scale optimization of JJCB medium was undertaken. Because of observed ammonia limitation, increased amounts of various existing medium components supplying inorganic or organic nitrogen were used, but these higher levels did not delay the onset of production significantly. This supplementation strategy, along with midcycle additions of 50 vol% glycerol to extend the fermentation, was implemented to increase cell mass and thus volumetric

Table 12
Summary of *A. unguis* Fermentations

Cultivation	Attribute	Midcycle glycerol additions	Peak titer (g/L)	Time (h)
Complex medium	All similar	None	0.6–0.9	300
Run 1: base medium of 7.6 g/L ardamine pH	All similar	None	1.0–1.25	214
Run 2: base medium of 7.6 g/L ardamine pH	Control	Four	2.8–2.9	441
	15.2 g/L ardamine pH		6.0	422
	5.6 g/L ammonium sulfate		3.6	441
	5.6 g/L maltoline		3.9	393
	11.2 g/L ammonium sulfate		3.9	465
Run 3: base medium of 15.2 g/L ardamine pH	Control (Rushton impeller)	Six	5.0–6.75	509
	Control (Maxflo T impeller)		9.1	509
	5.6 g/L ammonium sulfate		9.5	509
	5.6 g/L maltoline		8.3	509
	5.6 g/L each of ammonium sulfate and maltoline		9.15	509

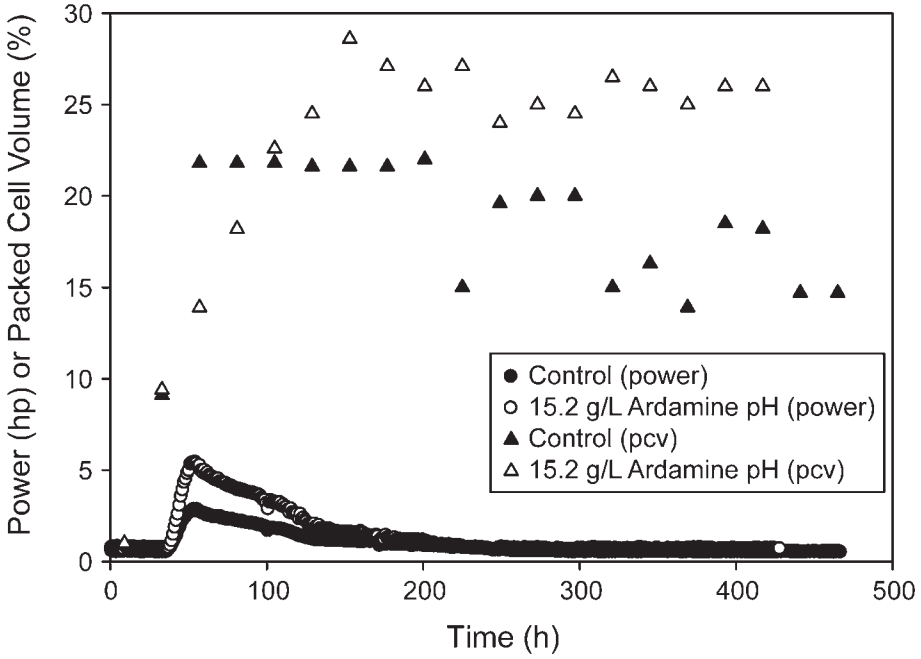

Fig. 1. Comparison of power draw and packed-cell volume for control and 15.2 g/L ardamine pH cultivations of *A. unguis* at 600-L pilot scale.

productivity. For the 15.2 g/L ardamine pH cultivation, the peak power draw at 80 h was nearly twofold higher than that of the control (7.6 g/L of ardamine pH) to maintain the DO at 50%sat despite a lower packed-cell volume (Fig. 1), indirectly suggesting a higher broth viscosity owing to possibly a more filamentous morphology. (Direct microscopic observations of morphology and viscosity measurements were not performed.) OURs peaked at 10–14 mmol/(L·h), and increases in titer were dramatic (Table 12, run 2).

Sharp rises in DO occurred at times ranging from 220 to 300 h and were earliest for the 15.2 g/L ardamine pH and 5.6 g/L ammonium sulfate fermentations, suggesting earlier depletion of critical nutrients in these cultivations. Broth pH for the 5.6 g/L maltoline and 5.6 g/L ammonium sulfate cultivations declined from 6.2 to 4.5 initially, at which point it then was controlled using base addition, suggesting organic acid production owing to carbon substrate metabolism. By contrast, the broth pH of control cultivations declined to 5.2, then increased to 6.0–6.4 with minimal uptake of acid or base for the duration of the fermentation.

Optimization of JJCB medium was continued using 15.2 g/L of ardamine pH in the new base medium despite higher observed peak power draw. The control cultivations with Rushton impellers reached 5.0 and 6.75 g/L, and the cultivation with the hydrofoil Maxflo T impeller *(60)* reached about 9.15 g/L (Table 12, run 3). This suggests some possible effect of shear or mixing on titer, as well as some reproducibility issues associated

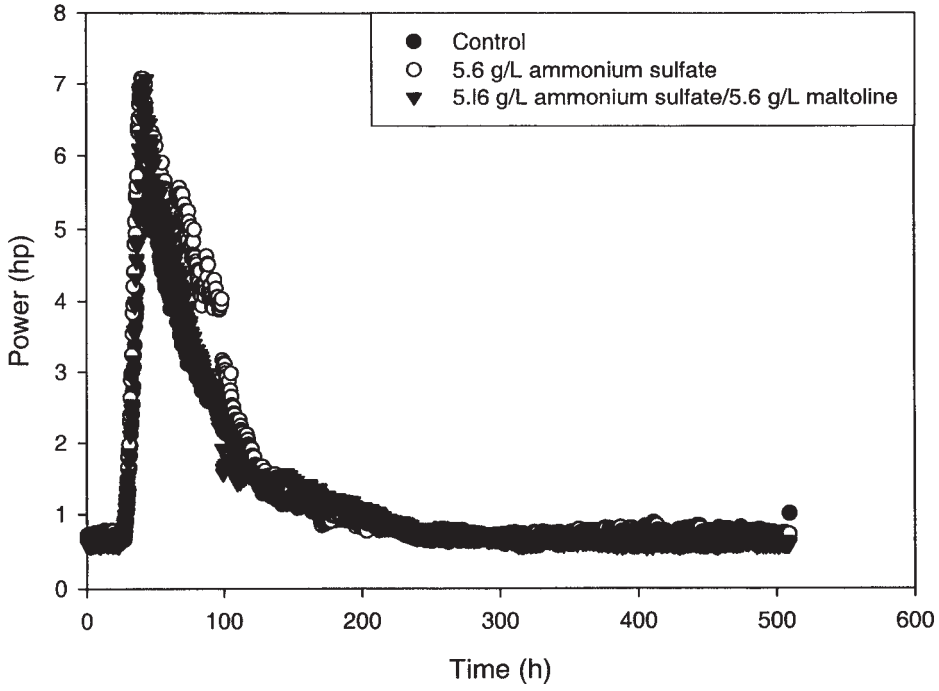

Fig. 2. Comparison of power draw for control, 5.6 g/L ammonium sulfate, and 5.6 g/L ammonium sulfate/5.6 g/L maltoline-supplemented cultivations (all containing 15.2 g/L of ardamine pH) of *A. unguis* at 600-L pilot scale.

with similar cultivation conditions at higher ardamine pH levels. Further characterization of performance may be needed during subsequent runs so that the impact of additional process development changes, such as optimal ammonium sulfate supplementation, may be more clearly apparent.

OURs increased twofold and ranged from 17 to 24 mmol/(L·h) with no clear relationship between OUR peak and subsequent performance. Dramatic drops in OUR/CER were evident when glycerol was exhausted and returned to prior values promptly after the addition of glycerol. The sharp rise in DO occurred from 230 to 290 h and was different for various supplemented media. For the 5.6 g/L maltoline case, DO remained at the control point of 50%sat until harvest. For the lowest producing control batch, it was delayed until 380 h, compared with 260 and 290 h for the other two control batches, suggesting a change in metabolism that was less favorable to production.

During control fermentations (after the initial characteristic sharp decline, rise, and fall), pH declined from 6.0 to 5.0, and there was little caustic/acid uptake. For the 5.6 g/L ammonium sulfate and 5.6 g/L ammonium sulfate/5.6 g/L maltoline cultivations, initial pH declined to 4.5, and the culture consumed caustic for the duration of fermentation, presumably owing to organic acid production. Peak power draws were similar among these production batches (Fig. 2), suggesting that further variations in

concentrations of non-ardamine pH medium components did not notably influence culture viscosity.

S. araneosa

Initial scale-up was conducted at the 600-L scale using fermentors with Maxflo T hydrofoil impellers. OURs reached 8–10 mmol/(L·h) at 50 h and declined to 5–7 mmol/(L·h) by harvest. Broth pH decreased from 7.2 to 6.7 by 40 h, rose to 7.2 by 50 h, then decreased to 5.3 by 200 h, and rose to 5.9 by 230 h. This behavior indicated several switches in metabolism. Titers of 411 and 531 mg/L were achieved at 277 h.

Based on this single initial run of only two fermentors and pressing material requirements, scale-up to the 15,000-L scale using A315 hydrofoil impellers was accomplished to provide larger quantities of sordarin. The decrease in broth pH to 5.3 was more substantial than that observed at the 600-L scale, most likely owing to higher CO_2 accumulation. Similarly to the 600-L scale production pattern, the 15,000-L scale titer reached about 300 mg/L by 120 h. In contrast to the 600-L scale, it did not increase further but, rather, remained stable at cultivation conditions at 294 ± 15 mg/L (5% relative standard deviation [rsd] over 13 daily samples) until harvest at 433 h. Potential reasons for this slightly lower titer on scale-up were not investigated; instead, the focus of development shifted toward improving titer at the 600-L scale.

Subsequent 600-L production fermentations were conducted a few years later that reproduced or even exceeded earlier titers. Peak OURs ranged from 4 to 11 mmol/(L·h) by 100–130 h, remained steady from 130 to 150 h, then declined slowly by 30% by harvest. RQs rose to 1.0 by 25 h, then remained at that value, indicating that sufficient glucose was available and utilized as a carbon source. For fermentations using Maxflo T hydrofoil impellers *(60)*, cell solids rose to 30–40 wt% by 60–80 h, remained constant through 200 h, then increased further to 45–60% by 280 h, most likely owing to a morphological change during the latter portion of the fermentation (Fig. 3). Production-phase-hydrolyzed sordarin titers reached 390 ± 35 mg/L (9.0%rsd for three cultivations) at 170 h and 360 ± 32 mg/L (8.9%rsd for three cultivations) at 280 h for all fermentations conducted using hydrofoil Maxflo T impellers *(60)*. Titers were less uniform for differing impeller geometries (586 ± 122 mg/L, 20.8%rsd and 1085 ± 177 mg/L, 16.3%rsd), suggesting an effect of impeller type (Rushton, CD-6, and HE-3; *[60]*) on cultivation performance.

Simple medium supplementation, specifically doubling concentrations of nitrogen sources (yeast extract 106 and monosodium glutamate) separately and together to increase cell mass, did not increase titers. Experiments to simplify the medium formulation were successful without adversely affecting titer. Their focus was to lower the unusually high concentration of insoluble calcium carbonate from 7.2 to 1.8 g/L to minimize postbatch cleaning steps caused by this inorganic salt forming a white residue on fermentor internal surfaces.

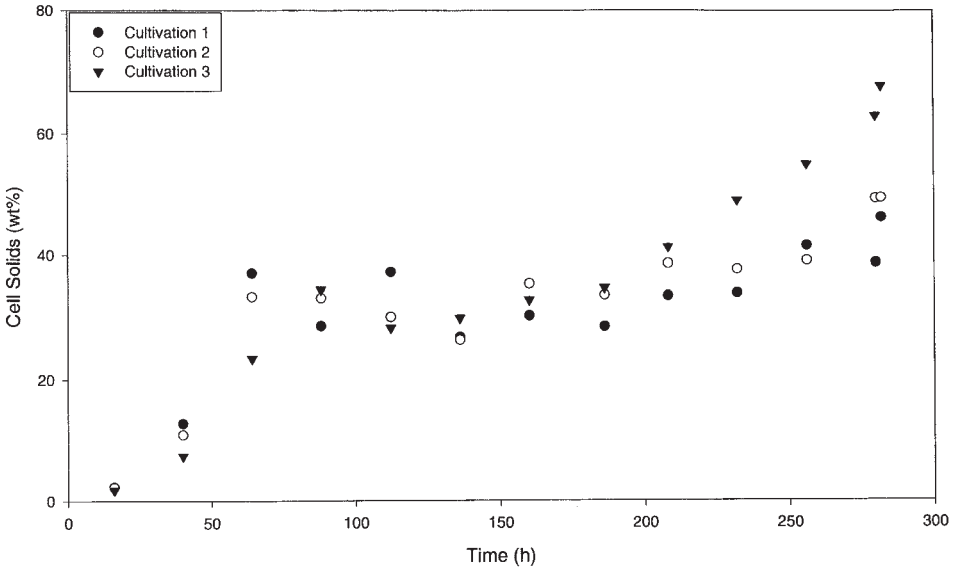

Fig. 3. Cell solids profile for three similar cultivations of *S. araneosa* at 600-L pilot scale.

Table 13
Summary of *S. roseoflavus* Fermentations

Cultivation	Attribute	Peak titer (mg/L)	Time (h)
Run 1	Seed OUR of 7 mmol/(L·h)	112–155	108
	Seed OUR of 14 mmol/(L·h)	89–114	108
Run 2	Seed OUR of 6 mmol/(L·h)	103–106	138
	Seed OUR of 6 mmol/(L·h), production agitation rate of 200 rpm	96	152
	Seed OUR of 6 mmol/(L·h), production pH of 5.0	40	152
	Seed OUR of 19 mmol/(L·h)	66–97	126–138

OUR, oxygen uptake rate

S. roseoflavus

Two similar sets of production cultivations were performed to meet material requirements (Table 13). For both runs, OURs reached an initial peak of 30 mmol/(L·h) at 20 h, remained stable, reached a second peak at 40 h of 30–40 mmol/(L·h), and then declined to 2 mmol/(L·h) by 120 h. Glucose was exhausted by 50–60 h, earlier in run 1 than the run 2 controls. This higher run 1 glucose uptake rate was likely related to cell solids reaching 80–90 wt% at 60 h in run 1, but only 10–30 wt% at 80 h in run 2. The average peak titer for run 1 was 122 ± 23 mg/L (18.8%rsd) at 108–131 h, and that of run 2 (excluding the pH 5.0 experiment in which growth was delayed and titer was notably lower) was 94 ± 16 mg/L (17.0%rsd) at

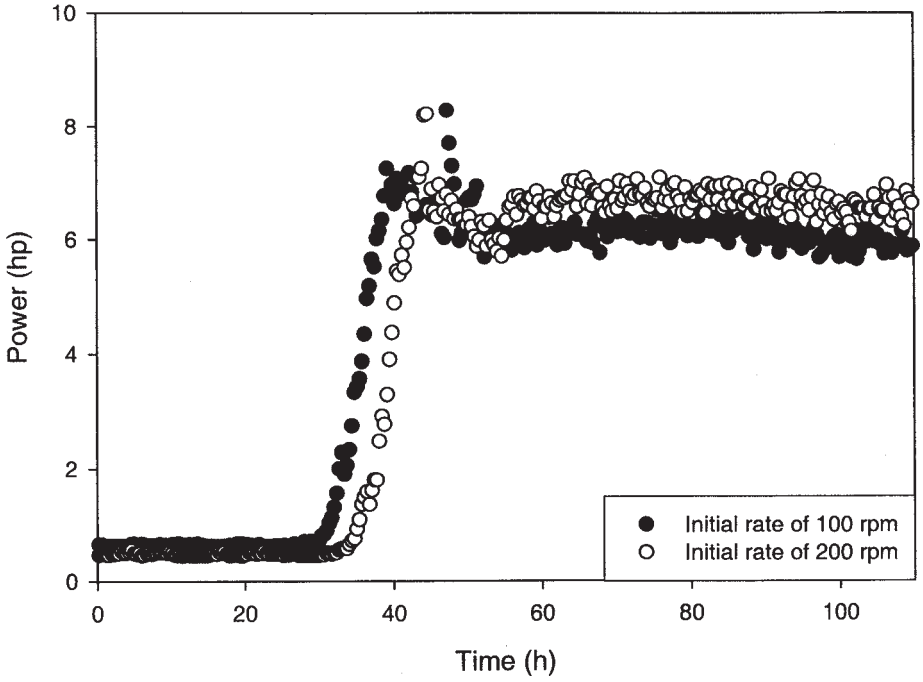

Fig. 4. Comparison of peak power draw for cultivations of *S. roseoflavus* with initial agitation rates of 100 vs 200 rpm (hydrofoil Maxflo T impellers).

126–152 h, about 1 d later than run 1. Titer variations within a run could not be attributed to seed fermentor OURs at the time of production fermentor inoculation, but they might have been affected by impeller-type variations (Rushton, CD-6, HE-3, Maxflo T, A315; *[60]*). Broth pH sensor problems, experienced toward the end of the cycle in several fermentations, were likely caused by soluble starch coating of glass pH sensors since "failed" probes worked acceptably after being removed from service and cleaned.

Although DO control was most active between 24 and 48 h, the key challenge noted from initial experimental batches was to minimize power draw between 2 and 5 d to reduce fermentor motor loads, preventing overheating. There was no significant effect of higher initial agitation rate using a hydrofoil Maxflo T impeller (100 vs 200 rpm) on titer or subsequent peak agitator power draw (Fig. 4), although peak titer may have been delayed slightly for the higher initial agitation rate (Table 13, run 2). Although peak power draw was not significantly affected, the stage of the seed-tank inoculum affected subsequent broth consistency of samples. When the seed fermentor OUR was 7.0 mmol/(L·h), production broth samples became gelatinous by 60 h. When the seed fermentor OUR was 14 mmol/(L·h), broth samples were only beginning to become gelatinous by 84 h. Titer ranges were somewhat lower when seed fermentors with higher OURs were utilized. For this process, efforts to alter viscosity through the hydrodynamic environment or inoculum stage were only partially effective.

Further efforts in medium development may be more likely to have a significant impact.

C. affinis

As with *S. roseoflavus*, two similar sets of cultivations were performed to meet material requirements. For BB1 medium with 14.4 g/L of Pharmamedia, OURs rose to 14 to 15 mmol/L by 100 h, then declined slowly until harvest. For BB1 medium with 7.2 g/L of Pharmamedia, OURs were slightly lower, ranging from 9 to 13.5 mmol/(L·h). Broth pH rose initially from 5.9 to 6.2, where it was controlled via acid addition, indicating likely amino acid consumption. Then when OUR peaked, broth pH declined sharply to 4.5, where it was controlled by base addition, indicating likely carbon source consumption. It subsequently rose sharply to 6.2, suggesting a return to amino acid consumption. In some batches, pH declined to 4.5, began taking base, and even returned to 6.2 again. This behavior suggested a switch between organic acid and ammonia production.

Using 14.4 g/L of Pharmamedia in BB1 medium, broths became so thick at about 120 h that airflow rates through the vessel were reduced and maximum tank operating conditions could not aerate and mix effectively. After removal of some broth to a drum (broth thickness clogged sewer piping), a 15 vol% sterile water shot was delivered to dilute the broth. The dilution was ineffective and the cultivation was terminated at 154 h. Titers were 25, 48.5, and 46 mg/L (39.8 ± 12.9 mg/L, 32%rsd). Subsequently, BB1 production medium was altered by reducing the Pharmamedia content by 50 wt% (from 14.4 to 7.2 g/L) to lower broth viscosity. For these 600-L cultivations, a 15 vol% sterile water shot added at 136 h was sufficient for dilution, and cultivations were harvested at 324–413 h. Although the average titer was 20.4 ± 7.4 mg/L (36%rsd), which was lower than with higher Pharmamedia fermentations, these 600-L fermentations were substantially easier to agitate, were amenable to straightforward isolation, and provided a useful basis for future process development efforts. The relatively high rsds for both sets of cultivations were caused partly by biologic variability and partly by different impeller types (first set: Rushton, Maxflo T; second set: Rushton, Maxflo T, A315, CD-6; *[60]*).

Conclusion

Challenges in early phase secondary metabolite production by fungal or filamentous bacterial cultures have been examined in relation to culture pelleting and morphology, particularly in shake-flask and fermentor seed stages. Key laboratory to pilot plant scale-up issues also were identified. For these early phase processes, synthesis pathways and large-scale cultivation usually are not well studied. Initial titers typically are low, at 50–200 mg/L, although there are some exceptions. Scale-up to the 600-L pilot scale from the 20- to 30-L laboratory scale (about 20- to 30-fold) to generate initial material is aimed at achieving or exceeding these titers, as well as

generating a reasonable large-scale process in a timely manner from which to base further development efforts. Eventual scale-up within the pilot plant from the 600-L to 15,000-L scale (about 25-fold) occurs as the compound's material requirements escalate.

Based on experiences presented during early phase process development for five model cultivations, key elements useful to consider for a successful initial pilot plant scale-up are as follows:

1. Minimization of culture pelleting and morphology, particularly in shake-flask and fermentor seed stages, all the way back to initial frozen seed vial preparation.
2. Transition of laboratory-scale media into media amenable to scale-up containing few if any solids, defined components if possible, and readily commercially available bulk nutrients.
3. Development of a medium that supports a broth possessing a reasonable viscosity.
4. Translation of laboratory-scale operating conditions to the pilot scale.
5. Establishment of reproducibility for similar fermentation vessels (within ±20% if possible) to fully evaluate potential benefits of changes in medium or process.
6. Evaluation of on-line and off-line data to determine which parameters are key to improved process performance, relating of on-line to off-line changes where possible to minimize laboratory sample throughput (i.e., relating of RQ to carbon source utilization, comparison of power draws for fermentors with equivalent geometries to indirectly assess the effect of medium components on viscosity, relating of broth pH to shifts in metabolism).

Although each of these goals can be incorporated into initial fermentation scale-up efforts for prompt and generally successful achievement of laboratory titers, the first one—broth pelleting and morphology in the seed stages—strongly influences the outcome of the remaining five.

Several potential differences are observed between the laboratory and pilot plant scales. Dissolved CO_2 tends to increase on scale-up owing to lower volumetric aeration rates, which may reduce productivity and peak titer or alter analog (structurally similar byproducts) ratios. Higher carbon utilization rates may be observed at the pilot scale, prompting midcycle nutrient additions to achieve similar laboratory-scale titers. Shear caused by both the agitation rate and impeller geometry can have a profound effect in seed as well as production fermentors. Most important, broth morphology influences broth thickness, which not only affects mixing but also aeration resistance and coating of instrument sensors. Identification and consideration of these factors, particularly shear, in developing scale-up conditions and interpreting scale-up behavior can be extremely beneficial for fungal and filamentous bacterial cultivations.

Appendix

A. niger

Seed flask medium (KF): 5.0 g/L of corn steep powder (Marcor, Carlstadt, NJ), 40.0 g/L of tomato paste (Contadina, San Francisco, CA), 10.0 g/L of oat flour (Quaker Oats, Chicago, IL), 10.0 g/L of glucose, and 10.0 mL/L of trace element solution (containing 1.0 g/L of $FeSO_4 \cdot 7H_2O$, 1.0 g/L of $MnSO_4 \cdot H_2O$, 0.2 g/L of $ZnSO_4 \cdot 7H_2O$, 0.1 g/L of $CaCl_2 \cdot 2H_2O$, 0.019 g/L of $NH_4MoO_4 \cdot 4H_2O$, 0.025 g/L of $CuCl_2 \cdot 2H_2O$, 0.056 g/L of H_3BO_3, and acidified with HCl) *(54)*. Presterilization pH was 6.8.

Seed fermentor medium (YME): 3.6 g/L of yeast extract (autolyzed code 106; BioSpringer USA, Minneapolis, MN), 7.2 g/L of maltoline ME (nondiastatic malt extract; Ingredient Technology, Mahwah, NJ), 1.8 mL/L of P2000 (polypropylene glycol 2000; Dow, Freeport, TX), and 25 g/L of cerelose (glucose monohydrate, added poststerilization; Corn Products, Argo IL). Presterilization pH was 7.0.

Production medium (JJCB without trisodium citrate): 2.8 g/L of yeast extract (autolyzed ardamine pH; Champlain Industrial, Clifton, NJ; currently available as Amberex pH from Sensient Flavors, Indianapolis, IN), 2.7 g/L of monopotassium phosphate, 2.7 g/L of maltoline ME, 2.7 g/L of ammonium sulfate, 2.7 g/L of P2000, 70 g/L of mannitol, and 20 g/L of cerelose added poststerilization. Presterilization pH was 6.0.

Seed fermentor conditions: temperature of 22–25°C, airflow rate of 150 L/min (0.83 vvm), back pressure of 0.7 kg/cm^2, and agitation rate of 100 rpm.

Production conditions: temperature of 22–35°C, back pressure of 0.7 kg/cm^2, agitation rate of 200–350 rpm (with higher agitation rates examined to promote pelleting and reduce the "caking" phenomenon around the sparger outlet), and airflow rate of 200–300 L/min (0.33–0.5 vvm) (with higher airflow rates also suspected of helping avoid "caking").

Off-line analysis: Cell solids (wt%) were measured by weighing the pellet after centrifugation (1500 rpm, 405*g*, 15 min) of 10 mL of whole broth. Titer was measured by extracting 75 mL of whole broth with 1.2 vol of methyl ethyl ketone (MEK)/g of broth (based on broth weight to avoid volume measurement error owing to gas holdup) and shaking horizontally for 1 h. After centrifugation (4000 rpm, 2880*g*, 20 min), the cell-free layer was evaporated to dryness and then redissolved in methanol for high-performance liquid chromatography (HPLC) analysis using a C8 column. Product was more than 95% cell associated. Glucose was analyzed using standard HPLC methods (Bio-Rad Aminex HPX-87H organic acid analysis column). Mannitol was not assayed.

A. unguis

Seed fermentor medium (YME): 3.8 g/L of yeast extract code 106, 7.6 g/L of maltoline ME, 1.9 mL/L of P2000, and 14 g/L of cerelose (added poststerilization). Presterilization pH was 7.0.

Production medium (JJCB): 7.6 g/L of ardamine pH, 2.8 g/L of monopotassium phosphate, 2.8 g/L of maltoline ME, 2.8 g/L of ammonium sulfate, 6.0 g/L of trisodium citrate, 2.8 g/L of P2000, 69 g/L of glycerol, and 18.7 g/L of cerelose (added poststerilization).

Seed fermentor conditions: temperature of 23.5°C, airflow rate of 100 L/min (0.55 vvm), back pressure of 0.7 kg/cm^2, and agitation rate of 100 rpm.

Production conditions: temperature of 23.5°C, airflow rate of 100–250 L/min (0.16–0.42 vvm), back pressure of 0.7–1.5 kg/cm^2, and agitation of 100–350 rpm, all cascaded to control DO at 50% saturation.

Off-line analysis: Cell solids (vol%) were estimated by measuring the pellet volume after centrifugation (1500 rpm, 405*g*, 15 min) of 10 mL of whole broth. Titer was measured by extracting 75 mL of whole broth with 1.2 mL of MEK/g of broth and shaking horizontally for 1 h. After centrifugation (4000 rpm, 20 min), the cell-free layer was evaporated to dryness, then redissolved in acetonitrile for HPLC analysis using a Phenomenex Aqua 5 µ C-18 column. Glucose and glycerol were analyzed using standard HPLC methods (Bio-Rad Aminex HPX-87H organic acid analysis column). Ammonia and phosphorus were measured using Ektachem slides (DT-60; Kodak, Rochester, NY) after dilution with deionized water to be in an appropriate analyzer range.

S. araneosa

Seed fermentor medium (AD-2): 23.6 g/L of glycerol, 3.7 g/L of yeast extract code 106, 0.93 g/L of sodium nitrate, 2.8 g/L of monosodium glutamate, 0.46 g/L of disodium phosphate, 0.93 g/L of magnesium sulfate heptahydrate, 0.93 mL/L of P2000, 0.93 mL/L of trace element solution (5.8 g/L of $FeCl_3 \cdot 6H_2O$, 0.1 g/L of $MnSO_4 \cdot H_2O$, 0.02 g/L of $CoCl_2 \cdot 6H_2O$, 0.015 g/L of $CuSO_4 \cdot 5H_2O$, 0.012 g/L of $NaMoO_4 \cdot 2H_2O$, 0.02 g/L of $ZnCl_2$, 0.005 g/L of $SnCl_2 \cdot H_2O$, 0.01 g/L of H_3BO_3, and 0.02 g/L of KCl), 7.4 g/L of calcium carbonate (added after pH adjustment), and 28 g/L of cerelose (added poststerilization). Presterilization pH was 7.0.

Production medium (AD-2): Same composition as seed fermentor medium except 166 g/L of cerelose (added poststerilization).

Seed fermentor conditions: temperature of 25°C, airflow rate of 100 L/min (0.55 vvm), back pressure of 0.7 kg/cm^2, and agitation rate of 100 rpm.

Production conditions: 600-L scale, temperature of 25°C; airflow rate of 100 L/min (0.83 vvm) back pressure of 0.7 kg/cm^2; and agitation rate of 100 rpm cascaded to control DO at 50%sat.

Off-line analysis: Cell solids (vol%) were estimated by measuring the pellet volume obtained by centrifugation (1500 rpm, 405*g*, 15 min) of 10 mL of whole broth. Broth titer was measured by extracting 75 mL of whole broth with 1.2 mL MEK/g of broth and shaking horizontally for 1 h. After centrifugation (4000 rpm, 2880*g*, 20 min), the cell-free layer was evaporated to dryness, then redissolved in methanol/water with NaOH added to a concentration of 0.1 *N*. Alternatively, 1:1 methanol:broth extractions were done, then adjusted to 0.1 *N* by NaOH addition. Because sordarin often

esterified during fermentation, broth extracts were hydrolyzed prior to analysis followed by acidification with H_2SO_4 to pH 7.0 to obtain reproducible results. After centrifugation, titers were quantified by analytical reverse-phase HPLC on either a Phenomenex Primesphere C8 or a Restek Ultra C8 column.

S. roseoflavus

Seed fermentor medium (ISM-3): 14.2 g/L of yeast extract 106, 9.4 g/L of maltoline ME, 0.47 g/L of magnesium sulfate heptahydrate, 0.028 g/L of ferric chloride hexahydrate, 0.94 mL/L of P2000, and 26 g/L of cerelose (added poststerilization).

Production medium (FR23): 30 g/L of partially hydrolyzed soluble starch (Stadex 60; A.E. Stalely, Tate and Lyle, Decatur, IL), 5.5 g/L of cerelose (sterilized together), 7.5 g/L of sucrose, 2.5 g/L of Amberex pH, 20 g/L of Pharmamedia (cottonseed flour; Traders Protein, Memphis, TN), and 3 mL/L of P2000. Soluble starch was dissolved in warm water during media batching.

Seed fermentor conditions: temperature of 28°C, airflow rate of 100 L/min (0.55 vvm), back pressure of 0.7 kg/cm^2, and agitation ramped from 100 to 130 rpm to control DO at 50%sat to obtain higher seed-tank OURs.

Production conditions: temperature of 28°C, airflow rate of 150–500 L/min (0.25–0.83 vvm), back pressure of 0.7–1.5 kg/cm^2, and agitation of 100–300 rpm with airflow and agitation rates each cascaded to control DO at 50%sat.

Off-line analysis: Cell solids (wt%) were measured by weighing the pellet obtained from centrifugation (4000 rpm, 2880*g*, 20 min) of 10 mL of whole broth. Titer was measured by fourfold extraction of 75 mL of whole broth with methanol, shaking horizontally for 30 min, centrifuging (4000 rpm, 2880*g*, 20 min), and analyzing the cell-free layer by HPLC using a Waters Symmetry C8 column. Glucose was analyzed using an enzymatic assay (YSI, Yellow Springs, OH).

C. affinis

Seed fermentor medium (LYCP-5): 8.4 g/L of potassium phosphate monobasic, 4.7 g/L of yeast extract 106, 9.3 g/L of Pharmamedia, 1.9 mL/L of 88% lactic acid, 0.93 mL/L of trace element solution (10.0 g/L of $FeSO_4 \cdot 7H_2O$, 10 g/L of $MnSO_4 \cdot H_2O$, 2.0 g/L of $ZnSO_4 \cdot 7H_2O$, 1.0 g/L of $CaCl_2 \cdot 2H_2O$, 0.19 g/L of $NH_4MoO_4 \cdot 4H_2O$, 0.25 g/L of $CuCl_2 \cdot 2H_2O$, 0.56 g/L of H_3BO_3, and acidified with HCl to ensure dissolution), 1.9 mL/L of P2000, and 30.3 g/L of cerelose (added poststerilization). Presterilization pH was 6.0.

Production medium (BB1): 72 g/L of mannitol, 7.2 or 14.4 g/L of Pharmamedia, 4.8 g/L of yeast extract 106, 3.8 g/L of L-glutamic acid, and 0.96 mL/L of P2000. Presterilization pH was 6.0.

Seed fermentor conditions: temperature of 22°C, airflow rate of 100 L/min (0.55 vvm), back pressure of 0.7 kg/cm^2, and agitation cascaded from 100 to 200 rpm to control DO at 50%sat.

Production conditions: temperature of 22°C, airflow rate of 125–1000 L/min (0.083–0.67 vvm) for 1500-L scale or 100–500 L/min (0.17–0.83 vvm) for 600-L scale, back pressure of 0.7–1.0 kg/cm^2, and agitation rate of 100–230 rpm for 1500-L scale and 100–300 rpm for 600-L scale (with airflow and agitation rates each cascaded to control DO at 50%sat). Airflow rates were sufficiently high to reduce power draw for higher agitation rates.

Off-line analysis: Broth titer was measured by 1:1-fold extraction of 75 mL of whole broth with acetone, shaking horizontally for 60 min, centrifuging (4000 rpm, 2880*g*, 20 min), and analyzing the cell-free layer by HPLC using a YMC Combiscreen C8 column.

References

1. Jones, P., Moore, D., and Trinci, A. P. J. (1988), *J. Gen. Microbiol.* **134,** 235–240.
2. Calam, C. T. (1976), *Process Biochem.* **11(7),** 7–12.
3. Gehrig, I., Bart, H.-J., Anke, T., and Germerdonk, R. (1998), *Biotechnol. Bioeng*. **59(5),** 525–533.
4. Hotop, S., Möller, J., Niehoff, J., and Schügerl, K. (1993), *Process Biochem.* **28,** 99–104.
5. Pallerla, S. and Chambers, R. P. (1997), *Bioresour. Technol.* **60(1),** 1–8.
6. Metz, B. and Kossen, N. W. F. (1977), *Biotechnol. Bioeng.* **19,** 781–799.
7. Schugerl, K., Wittler, R., and Lorenz, T. (1983), *Trends Biotechnol.* **1(4),** 120–123.
8. Whitaker, A. and Long, P. A. (1973), *Process Biochem*. **11,** 27–31.
9. Whitaker, A. (1992), *Appl. Biochem. Biotechnol.* **32,** 23–35.
10. Lawton, P., Whitaker, A., Odell, D., and Stowell, J. D. (1989), *Can. J. Microbiol*. **35,** 881–889.
11. Papagianni, M. (2004), *Biotechnol. Adv.* **22,** 189–259.
12. van Suijdam, J. C., Kossen, N. W. F., and Paul, P. G. (1980), *Eur. J. Appl. Microbiol. Biotechnol*. **10,** 211–221.
13. Jüsten, P., Paul, G. C., Nienow, A. W., and Thomas, C. R. (1996), *Biotechnol. Bioeng*. **52,** 672–684.
14. Bujalski, W., Cox, P. W., Thomas, C. R., Nienow, A. W., Preide, M. A., and Viesturs, U. E. (1997), in *Bioreactor/Process Fluid Dynamics*, BHR Group Conference Series, Publication No. 25, Nienow, A. W., ed., Mechanical Engineering Publications, London, pp. 9–25.
15. Tucker, K. G. and Thomas, C. R. (1994), *Biotechnol. Techniques* **8(3),** 153–156.
16. Braun, S. and Vecht-Lifshitz, S. E. (1991), *Trends Biotechnol.* **9,** 63–68.
17. Fujita, M., Iwahori, K., Tatsuta, S., and Yamakawa, K. (1994), *J. Ferment. Bioeng*. **78(5),** 368–373.
18. Smith, G. M. and Calam, C. T, (1980), *Biotechnol. Lett*. **2(6),** 261–266.
19. Tuttobello, R. and Mill, P. J. (1961), *Biochem. J*. **79,** 51–57.
20. Archer, D. B., MacKenzie, D. A., and Ridout, M. J. (1995), *Appl. Microbiol. Biotechnol*. **44,** 157–160.
21. Takahashi, J., Abekawa, G., and Yamada, K. (1960), *J. Agric. Chem. Soc. Japan* **34,** 1043–1045.
22. Elmayergi, H., Scharer, J. M., and Young, M. M. (1973), *Biotechnol. Bioeng*. **15,** 845–849.
23. Cui, Y. Q., van der Lans, R. G. J. M., and Luyben, K. C. A. M. (1997), *Biotechnol. Bioeng*. **55(5),** 715–726.
24. Cui, Y. Q., van der Lans, R. G. J. M., Giuseppin, M. L. F., and Luyben, K. C. A. M. (1998), *Enzyme Microb. Technol.* **23,** 157–167.
25. Moore, J. and Bushell, M. E. (1997), *Mycol. Res*. **101(10),** 1237–1241.

26 Sinha, J., Bae, J. T., Park, J. P., Kim, K. H., Song, C. H., and Yun, J. W. (2001), *Appl. Microbiol. Biotechnol*. **56,** 88–92.
27 Berovic, M., Cimerman, A., Steiner, W., and Koloini, T. (1991), *Appl. Microbiol. Biotechnol.* **34,** 579–581.
28 Vecht-Lifshitz, S. E., Sasson, Y., and Braun, S. (1992), *J. Appl. Bacteriol.* **72,** 195–200.
29 Belmar-Beiny, M. T. and Thomas, C. R. (1991), *Biotechnol. Bioeng*. **37(5),** 456–462.
30. Li, Z. J., Shukla, V., Wenger, K. S., Fordyce, A. P., Pedersen, A. G., and Marten, M. R. (2002), *Biotechnol Prog*. **18,** 437–444.
31. Snell, R. L. and Schweiger, L. B. (1949), US patent 2,492,667.
32. Schweiger, L. B. and Snell, R. L. (1949), US patent 2,476,159.
33. Martin, S. M. and Waters, W. R. (1952), *Ind. Eng. Chem*. **44,** 2229–2233.
34. Kubicek, C. P. and Röhr, M. (1977), *Eur. J. Appl. Microbiol. Biotechnol.* **4,** 167–175.
35. Kubicek, C. P. and Röhr, M. (1978), *Eur. J. Appl. Microbiol. Biotechnol.* **5,** 263–271.
36. Kubicek, C. P. and Röhr, M. (1985), *Appl. Environ. Microbiol.* **50(5),** 1336–1338.
37. Kubicek, C. P., Zehentgruber, O., El-Kalak, H., and Röhr, M. (1980), *Eur. J. Appl. Microbiol. Biotechnol*. **9,** 101–115.
38. Röhr, M., Zehentgruber, O., and Kubicek, C. P. (1981), *Biotechnol. Bioeng*. **23,** 2433–2445.
39. Nowakowska-Waszczuk, A., Rubaj, E., Matusiak, B., and Kosiek, E. (1984), *Appl. Microbiol. Biotechnol.* **20,** 416–418.
40. Röhr, M., Kubicek, C. P., Zehentgruber, O., and Orthofer, R. (1987), *Appl. Microbiol. Biotechnol*. **27,** 235–239.
41. Roukas, T. (1991), *J. Ind. Microbiol.* **7(30),** 221–225.
42. Yigitoglu, M. (1992), *J. Islamic Acad. Sci.* **5(2),** 100–106.
43. Kim, K. S., Yoo, Y. J., and Kim, M. H. (1995), *J. Ferment. Bioeng*. **79(6),** 555–559.
44. Campeanu, G., Pele, M., and Campeanu, M. (1998), *Roum. Biotechnol. Lett*. **3(3),** 193–200.
45. Wongwicharn, A., McNeil, B., and Harvey, L. M. (1999), *Enzyme Microb. Technol.* **24,** 489–497.
46. Blom, R. H., Pfeifer, V. F., Moyer, A. J., Traufler, D. H., Conway, H. F., Crocker, C. K., Farison, R. E., and Hannibal, D. V. (1952), *Ind. Eng. Chem*. **44(2),** 435–440.
47. Perlman, D. (1949), *Econ. Bot.* **3(4),** 360–374.
48. Prescott, S. C. and Dunn, C. G. (1959), in *Industrial Microbiology*, 3rd ed., Dunn, C. G., ed., McGraw-Hill, New York, pp. 533–577.
49. Lockwood, L. B. and Schweiger, L. B. (1967), in *Microbial Technology*, Peppler, H. J., ed., Reinhold Publishing, New York, pp. 183–199.
50. Takahashi, J., Hidaka, H., and Yamada, K. (1965), *Agric. Biol. Chem*. **29(4),** 331–336.
51. Buckland, B., Brix, T., Fastert, H., Gbewonyo, K., Hunt, G., and Jain, D. (1985), *Bio/Technology* **3,** 982–988.
52. Junker, B., Brix, T., Lester, M., Kardos, P., Adamca, J., Lynch, J., Schmitt, J., and Salmon, P. (2003), *Biotechnol. Prog*. **19,** 693–705.
53. Junker, B., Seeley, A., Lester, M., Kovatch, M., Schmitt, J., Borysewicz, S., Lynch, J., Zhang, J., and Greasham, R. (2002), *Biotechnol. Bioeng*. **79(6),** 628–640.
54. Dombrowski, A. W., Bills, G., Sabnis, G., Koupal, L., Meyer, R., Ondeyka, J., Giacobbe, R., Monaghan, R., and Lingham, R. (1992), *J. Antibiot.* **45,** 671–678.
55. Feighner, S. D., Salituro, G., Smith, J. L., and Tsou, N. N. (1994), US patent 5,350,763.
56. Bills, G., Dombrowski, A., Horn, W., Jansson, R., Rattray, M., Schmatz, D., and Schwartz, R. (1997), PCT Int. Appl. WO 9811891.
57. Shastry, M., Nielsen, J., Ku, T., Hsu, M.-J., Liberator, P., Anderson, J., Schmatz, D., and Justice, M. C. (2001), *Microbiology* **147,** 383–390.
58. Barrett, D. (2002), *Biochim. Biophys. Acta* **1587,** 224–233.
59. Royce, P. N. (1992), *Biotechnol. Bioeng*. **40,** 1129–1138.
60. Junker, B. H., Mann, Z., and Hunt, G. (2000), *Appl. Biochem. Biotechnol.* **89,** 67–83.
61. Johansen, C. L., Coolen, L., and Hunik, J. H. (1998), *Biotechnol. Prog*. **14,** 233–240.
62. Papagianni, M., Mattey, M., and Kristiansen, B. (1999), *Process Biochem.* **35,** 359–366.
63. Tamura, S., Park, Y., Toriyama, M., and Okabe, M. (1997), *J. Ferment. Bioeng.* **83(6),** 523–528.

64. Qazi, G. N., Gaud, C. N., Chaturvedi, S. K., Chopra, C. L., Trager, M., and Onken, U. (1990), *J. Ferment. Bioeng*. **69(1),** 72–74.
65. Tucker, K. G., Patel, D., and Thomas, C. R. (1994), *IChemE Res. Event* **1,** 107–109.
66. Mitard, A. and Riba, J. P. (1988), *Biotechnol. Bioeng.* **32,** 835–840.
67. Cui, Y. Q., van der Lans, R. G. J. M., and Luyben, K. C. A. M. (1998), *Biotechnol. Bioeng*. **57(4),** 409–419.
68. Tucker, K. G. and Thomas, C. R. (1992), *Biotechnol. Lett.* **14(11),** 1071–1074.
69. Paul, K. R., Paul, G. C., and Thomas, C. R. (1995), *IChemE Res. Event* **2,** 980–982.
70. Vecht-Lifshitz, S. E., Magdassi, S., and Braun, S. (1990), *Biotechnol. Bioeng.* **35,** 890–896.
71. Gomez, R., Schnabel, I., and Garrido, J. (1988), *Enzyme Microb. Technol.* **10,** 188–191.
72. Trinci, A. P. J. (1970), *Arch. Mikrobiol.* **73,** 353–367.
73. Paul, G. C. and Thomas, C. R. (1995), *IChemE Res. Event* **2,** 974–976.
74. Choi, D. B., Park, Y. S., and Okabe, M. (1998), *J. Ferment. Bioeng*. **86,** 413–417.
75. Pirt, S. J. and Callow, D. S. (1959), *Nature* **184(4683),** 307–310.
76. El-Enshasy, H., Hellmuth, K., and Rinas, U. (1999), *Appl. Biochem. Biotechnol.* **81(1),** 1–11.
77. Darby, R. T. and Mandels, G. R. (1954), *Mycologia* **46,** 276–287.
78. Choi, D. B., Park, E. Y., and Okabe, M. (2000), *Biotechnol. Prog*. **16,** 525–532.
79. Pera, L. M. and Callieri, D. A. (1999), *J. Microbiol. Biotechnol*. **15(5),** 647–649.
80. Chopra, C. L., Qazi, G. N., Chaturvedi, S. K., Gaind, G. N., and Atal, C. K. (1981), *J. Chem. Tech. Biotechnol*. **31,** 122–126.
81. Kobayashi, H. and Suzuki, H. (1972), *J. Ferment. Technol.* **50(12),** 835–843.
82. Clark, D. S. (1962), *Can. J. Microbiol.* **8,** 133–136.
83. Choudhary, A. Q. and Pirt, S. J. (1965), *J. Gen. Microbiol*. **41,** 99–107.
84. Qadeer, M. A. and Abdullah, J. S. (1971), *Pakistan J. Biochem*. **4(1),** 33–38.
85. Chen, Z.-W., Ku, C.-H., Weng, C.-J., and Chen, T.-L. (1997), *Appl. Biochem. Biotechnol.* **67,** 249–258.
86. Trinci, A. P. J. (1983), *Trans. Br. Mycol. Soc.* **81,** 408–412.
87. Olsvik, E., Tucker, K. G., Thomas, C. R., and Kristiansen, B. (1993), *Biotechnol. Bioeng*. **42,** 1046–1052.
88. Papagianni, M., Mattey, M., and Kristiansen, B. (1994), *Biotechnol. Lett.* **16(9),** 929–934.
89. van Suijdam, J. C. and Metz, B. (1981), *Biotechnol. Bioeng.* **23,** 111–148.
90. Galbraith, J. C. and Smith, J. E. (1969), *Trans. Br. Mycol. Soc*. **52(2),** 237–246.
91. Ho, C. S. and Smith, M. D. (1986), *Biotechnol. Bioeng*. **28(5),** 668–677.
92. McIntyre, M. and McNeil, B. (1997), *Enzyme Microb. Technol.* **21,** 479–483.
93. Kamal, K. P., Verma, U. M., Nag, A. K., and Singh, S. P. (1999), *Asian J. Chem*. **11(3),** 1020–1022.
94. Cox, P. W. and Thomas, C. R. (1994), *IchemE Res. Event* **1,** 82–84.
95. Vanags, J. J., Priede, M. A., Are, R. J., and Viesturs, U. E. (1992), *Proceedings of the Latvian Academy of Sciences*, no. 6, pt. B, Riga, Latvia, pp. 60–64.
96. Gyamerah, M., Merichetti, G., Adedayo, O., Scharer, J. M., and Moo-Young, M. (2003), *Appl. Microbiol. Biotechnol*. **60,** 403–407.