Developing an Industrial Artemisinic Acid Fermentation Process to Support the Cost-Effective Production of Antimalarial Artemisinin-Based Combination Therapies

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Artemisinin-based combination therapies (ACTs) are currently unaffordable for many of the people who need them most. A major cost component of ACTs is the plant-derived artemisinin. A fermentation process for a precursor to artemisinin might provide a viable second source to stabilize the artemisinin supply and therefore reduce price. The heterologous production of artemisinic acid, an artemisinin precursor, by Saccharomyces cerevisiae was improved 25-fold from a 100 mg/L flask process to a 2.5 g/L process in bioreactors. A defined medium fed-batch process with galactose as the carbon source and inducer was developed, with titers of 1.3 g/L. In this strain ERG9 was controlled with promoter Pmet3 so that methionine repressed the sterol biosynthesis pathway and increased precursor availability for artemisinic acid biosynthesis. Addition of methionine to the process increased artemisinic acid titers to 1.8 g/L. A dissolved oxygen-stat algorithm was developed, which simultaneously controlled the agitation and feed pump. This improved process control and increased titers to 2.5 g/L.
Keywords: artemisinic acid, ACT, Saccharomyces cerevisiae, fermentation, isoprenoid

Introduction

Malaria affects millions of people world wide and kills over a million people annually, primarily children under the age of five in developing countries.[1] Most treatments for this disease are increasingly ineffective because of the development of drug resistance in the malaria parasite *Plasmodium falciparum*.[2] Drugs synthesized from the plant-derived compound artemisinin are currently the best available therapies; however, the cost of these treatments often excludes those most in need. In addition, demand for the therapies is increasing faster than the compound can be produced from its native source, the plant *Artemisia annua*.[3] A consistent supply of any plant-derived therapy has inherent challenges, because of long production cycles and weather and climate-dependent product yields.

Drug production by fermentation has many advantages over agricultural production, including scalability, price, and supply flexibility. Recently, artemisinic acid, a precursor to artemisinin has been produced by *Saccharomyces cerevisiae* at the laboratory scale at a significantly higher specific productivity than *A. annua*.[4] A semisynthesis of artemisinin from the precursor molecule artemisinic acid could provide an attractive alternative source of the compound if high yields are obtained from bioreactors. A fermentation process was developed for the artemisinic acid producing *S. cerevisiae* strain Y51 to validate heterologous production of artemisinic acid in bioreactors as a potential supply route for inexpensive artemisinin.

Though many industrial *S. cerevisiae* processes incorporate undefined components, process development with Y51 was initiated with chemically defined media. Chemically defined media have several advantages. First of all, with defined media, it is much easier to monitor nutrient availability. Also, undefined raw materials can be significantly more variable than simple salts and sugars,[5] which can lead to supply issues during scale-up and routine production. Synthetic medium is inexpensive, an important consideration for an antimalarial drug. Finally, it is a good choice for Y51 since plasmid selection is dependent on a leucine auxotrophy,[4] and the concentration of leucine in undefined components can be unknown or variable.

The induction of key biosynthetic genes in *S. cerevisiae* Y51 required galactose. Although cell growth on glucose is faster and less costly, glucose in the medium represses galactose induction. A dissolved-oxygen-stat (DO-stat) algorithm was designed that simultaneously controlled agitation and galactose feed. With this algorithm, high-density production was possible in cultivations where glucose was used first for growth followed by a galactose feed for growth and induction. Fermentation processes were developed yielding cell densities of 120–200 OD_{600} and artemisinic acid titers as high as 2.5 g/L. The volumetric productivity for the complete process was 0.43 g/L/day, a significant improvement over prior reports (0.026 g/L/day[4]).

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Materials and Methods

Strains and media

S. cerevisiae Y51 was used for all studies. Y51 is derived from EPY224[4] and has the genotype *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 δ1::P_{GAL1}-tHMGR δ2::P_{GAL1}-UPC2-1 δ3::P_{GAL1}-tHMGR δ4::P_{GAL1}-ERG20 erg9::P_{MET3}-ERG9::* HIS3 [pAM179-LEU2d] where *δn::* denotes integration into different *δ* insertion sites. pAM179 is a high-copy 2*μ* LEU2d plasmid expressing amorphadiene synthase from the GAL1 promoter, amorphadiene oxidase from the GAL10 promoter and the cytochrome P450 reductase from the GAL1 promoter. Y51 is autotrophic for lysine and uracil.

Seed cultures for flasks and bioreactors were prepared by inoculating 1 mL of frozen cells in 20% (v/v) glycerol into a 250-mL flask containing 50 mL of seed medium. For flasks, the seed cultures were grown for 2 days at 30°C to an OD_{600} of 4–7, and used to inoculate production flasks at an OD_{600} of 0.05. For bioreactors, seed cultures were subcultured after ~24 h of growth at 30°C by transferring 2 mL of culture into additional 250 mL flasks containing 50 mL seed medium. These flasks were grown for 2 days at 30°C to an OD_{600} of 5–10. Two seed flasks were pooled and used to inoculate 900 mL of batch medium in a 2-L bioreactor (10% v/v).

The media used for this work were based on media described by van Hoek et al.[6] The trace metal solution contained (per L) EDTA 15 g, $ZnSO_4 \cdot 7H_2O$ 10.2 g, $MnCl_2 \cdot 4H_2O$ 0.50 g, anhydrous $CuSO_4$ 0.5 g, $CoCl_2 \cdot 6H_2O$ 0.86 g, $Na_2MoO_4 \cdot 2H_2O$ 0.56 g, $CaCl_2 \cdot 2H_2O$ 3.84 g, and $FeSO_4 \cdot 7H_2O$ 5.12 g. The vitamin solution contained (per L): biotin 0.05 g, calcium pantothenate 1 g, nicotinic acid 1 g, myoinositol 25 g, thiamine HCl 1 g, pyridoxol HCl 1 g, and *p*-aminobenzoic acid 0.2 g. The batch and seed medium contained (per L) uracil 0.2 g, lysine 5 g, $(NH_4)_2SO_4$ 15 g, KH_2PO_4 8 g, $MgSO_4$ 3 g, $ZnSO_4 \cdot 7H_2O$ 0.72 g, vitamin solution 12 mL, and trace metals 10 mL. Seed medium contained 25 g/L glucose. Carbon type and concentration in the batch medium are described in the results. Methionine (0.25 g/L) was included in the batch medium for processes in Figure 3 (methionine containing process) and Figures 5 and 6. All feed media contained (per liter) trace elements 10 mL, vitamins 12 mL, KH_2PO_4 9 g, g/L anhydrous $MgSO_4$ 2.5 g, K_2SO_4 3.5 g, Na_2SO_4 0.28 g, uracil 1 g, and lysine 5 g. For the glucose feed, carbon concentration was 585 g/L and for the galactose feed carbon concentration was 405 g/L. Methionine (1 g/L) was included in the feed medium for the process in Figure 4 (methionine containing process) and Figures 5 and 6.

The batch medium was modified for flask production with the addition (per liter) of glucose 2 g, galactose 18 g, methionine, 0.25 g, and succinate buffer (pH 5.0), 100 mM. Flasks were also run in SD-HIS-LUE-MET-TRP[4] with the addition (per liter) of lysine 30 mg, glucose 2 g, galactose 18 g, and methionine 1 mM.

Flask bioprocesses

Artemisinic acid production was tested in flasks in the modified SD medium and bioreactor batch medium. For both cases, the seed culture was grown the same medium as the production medium, except that the only carbon was glucose (20 g/L) and methionine was omitted. Both media were tested in triplicate. Production cultures (50 mL in a 250-mL baffled flasks) were incubated in a rotary shaker for 6 days at 30°C.

Fed-batch bioprocesses

Fermentations were in 2 L Applikon Bioconsole ADI 1025's with ADI 1010 controllers. The pH was automatically controlled at 5.0 with the addition of 9.9 M NH_4OH. Temperature was maintained at 30°C and airflow was supplied 1 LPM. Dissolved oxygen was maintained at 40% of saturation with agitation (300–1,200 rpm) and oxygen enrichment.

Two types of feeding strategies were employed during the fermentation development process. The first was a simple exponential feed triggered by an increase in dissolved oxygen upon depletion of sugars in the batch medium. The feed rate was determined by the following equation:

$$F = \mu_{set} S_0 \, e^{\mu_{set}(t-t_0)}$$

where F is the substrate mass flow rate (g/h), S_0 is the mass of substrate in the batch media (g), μ_{set} is the user defined specific feed rate (h^{-1}), t is the batch age (h), and t_0 is the batch age when the feed was initiated (h).

During the exponential growth phase, optical densities, ethanol concentrations, and galactose concentrations were measured off-line. When the specific growth rate began to decrease relative to the specific feed rate, or when ethanol and/or galactose began to accumulate, the feeds were adjusted as described in the text.

The second type of fed-batch fermentation feeding strategy employed was a DO-stat algorithm that simultaneously controlled agitation and feed. The algorithm is diagrammed in Figure 4. This algorithm was used subsequent to glucose delivery by a programmed feed or as the sole method of galactose delivery.

Glucose, acetate, and ammonia determination

Glucose, acetate, and ammonia were measured with a Bioprofile 300 analyzer (Nova Biomedical) according to the manufacturer's instructions. When concentrations were above the maximum assay range, samples were diluted with an appropriate volume of Nova buffer. Nova buffer contains (per L) LiCl 0.64 g, LiOH 0.46 g, and HEPES 6 g.

Galactose and ethanol determination

Galactose and ethanol were assayed using R-Biopharm (South Marshall, MI) kits numbered 10 176 303 035 and 1 176 290 035, respectively, according to the manufacturer's instructions. When concentrations were outside the assay range, the samples were diluted with water.

Artemisinic acid determination

Flask samples were extracted by combining 0.25 mL whole broth and 0.75 mL methanol with 0.1% formic acid. Bioreactor samples were extracted by combing 0.05 mL whole broth and 0.95 mL methanol with 0.1% formic acid. All samples were mixed for 30 min. Cell debris was removed by centrifugation (5 min at 14,000*g*). Samples were filtered through Microcon Ultracel YM-30 spin filters (Millipore) and analyzed by HPLC (Agilent 1200 with UV detection at 212 nm) with methanol/water with 0.1% formic acid for the mobile phase. Twenty microliters of sample were injected onto a Supelco Discovery C8 column (100 mm × 4.6 mm, 5.0 *μ*m 180 Å). The separation method started with

Table 1. Production of Artemisinic Acid in a 6-Day Shake Flask Fermentation

Medium	Artemisinic Acid (mg/L)	OD_{600}	Artemisinic Acid [mg/(L OD)]
SD	76 ± 20	2.4	31.7
Bioreactor batch medium	197 ± 40	9.3	21.2

0.5 min at 70% methanol, followed by a 6.2 min gradient up to 97% methanol. The methanol concentration was maintained at 97% for 0.3 min, then decreased back down to 70% over 0.5 min and held at that concentration for 2 min. The retention time of the artemisinic acid for this method was 6.3 min. Analytical standards were prepared using purified artemisinic acid.

Results and Discussion

Artemisinic acid production in flasks

Initial tests of artemisinic acid production by *S. cerevisiae* Y51 were undertaken in shake flasks (Table 1). The production process selected for bioreactors was adapted for a flask test by using bioreactor batch medium containing 20 g/L galactose and 50 mM succinate buffer pH 5.0. This medium was compared in flasks to SD medium, which had been used for production previously.[4] Production in SD medium was somewhat lower than previously reported (100 mg/L).[4] Production in bioreactor batch medium was more than double the production in SD medium primarily due to better cell growth in bioreactor batch medium (9.3 OD_{600} compared to 2.4 OD_{600}, Table 1). Production per OD was lower in bioreactor medium (Table 1), however, the flask data suggested that a Y51 process in bioreactors with this medium was feasible.

Characterization of a high-density, glucose fed-batch process with an artemisinic acid producing strain

To determine the maximum density achievable with the strain in a high-density process, *S. cerevisiae* Y51 was grown on glucose without the addition of galactose for induction. The strain achieved an OD_{600} of 250 in a defined medium with glucose as the carbon source (Figure 1). The fermentation batch phase began with 25 g/L glucose and was grown until the glucose was depleted (~18 h after inoculation), and then an exponential feed with an 8-h doubling time was initiated. The exponential feed continued for 34 h until it reached a rate of 30.4 g/h, then it was reduced to 15.2 g/h and held constant. The ethanol accumulated to 10 g/L during the first 48 h of the process; however the ethanol was consumed during the carbon restricted phase of the fermentation (Figure 1). Ammonium was maintained between 3 and 5 g/L by ammonium sulfate in the batch medium and the addition of ammonium hydroxide for pH control.

Artemisinic acid production in a high-cell density galactose fed-batch process

In strain Y51, key biosynthetic genes required galactose for induction. The presence of glucose in the medium represses galactose induction. The first approach for an induced Y51 process in bioreactors used galactose as carbon and inducer throughout all of the process, except for the initial growth phase. Seed cultures were grown on glucose, and the bioreactor started with 10 g/L glucose (in addition to 10 g/L galactose), however, the feed contained only galactose. While Y51 grew quickly and easily on glucose, growing a high-density culture using only galactose as a carbon source proved to be more challenging. Galactose was consumed at a much slower rate than glucose and consequently the culture growth rate was significantly slower. Flask experiments using galactose in seed medium suggested that the production strains doubled every 10 h on galactose (data not shown). However, during fed-batch fermentation, galactose accumulated during the exponential feed with set doubling times of 10 h (Figure 2). To prevent galactose accumulation in the process shown, the feed was turned off until the galactose concentration reached zero, then resumed with a 12-h doubling time. The maximum feed rate was 15.4 g/h and was reached ~54 h after initiation of the feed. Subsequently, the feed rate was changed to a constant feed rate of 10.3 g/h. The maximum OD reached was 110 and peak production of artemisinic acid was 1.3 g/L (Figure 2).

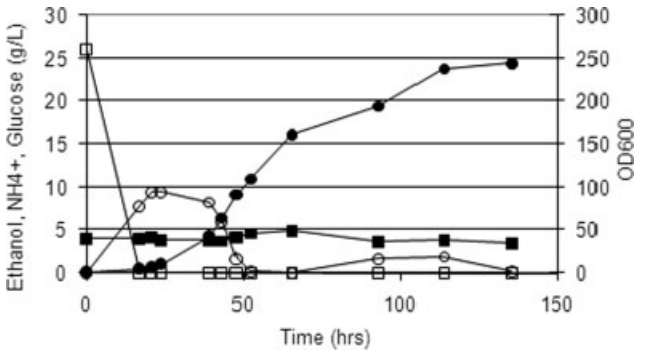

Figure 1. Growth of *S. cerevisiae* Y51 in a glucose limited fed-batch process without induction. Glucose (□), ethanol (○), NH_4^+ (■), and OD_{600} (●).

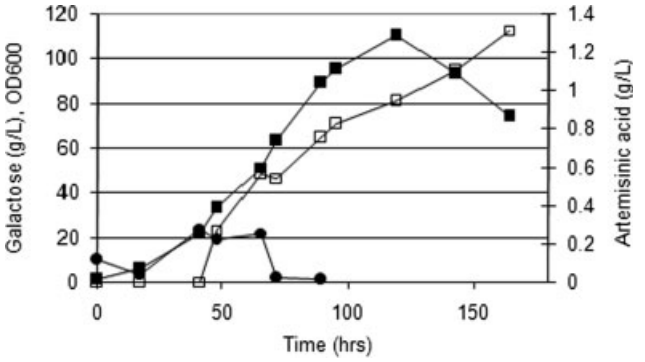

Figure 2. Cell growth and production of artemisinic acid by *S. cerevisiae* Y51 in a galactose fed-batch process.
Galactose (●), OD_{600} (■), and artemisinic acid (□).

The effects of reducing flux through competing isoprenoid pathways on the fermentation process

S. cerevisiae naturally produces sterols, which are synthesized from the same precursor, farnesyl pyrophosphate (FPP), as artemisinic acid. The enzyme that initiates the conversion of FPP to sterols is squalene synthase, which is encoded by the gene ERG9.[7] In strain Y51, a methionine repressible promoter (Pmet3) was used to downregulate ERG9 so that more FPP was available for synthesis of artemisinic acid.[4] Titers of amorpha-4,11-diene (the isoprenoid precursor of artemisinic acid) were doubled in flasks by using Pmet3 to regulate ERG9 and adding 1 mM (0.15 g/L) methionine

to the medium.[4] A galactose fed process was run in the same manner as described in Figure 2 to analyze the effects of methionine on artemisinic acid production in high-density bioreactors. The bioreactor contained 0.25 g/L methionine in the batch media and 1 g/L in the feed media. The bioreactor containing methionine achieved a maximum artemisinic acid concentration of 1.8 g/L while the bioreactor without methionine achieved a maximum artemisinic acid concentration of 1.3 g/L (Figure 3). Although the methionine increased production to 1.4 times the control, the improvement in high-density bioreactors was not as large as the two-fold improvement of amorpha-4,11-diene titers seen in flasks.[4]

Improvement in process control with a modified DO-stat feeding strategy for the galactose fed-batch fermentation

While the programmed exponential feed worked well for controlling glucose concentration and ethanol generation in high-density fermentation, it did not work well for maintaining low positive carbon concentrations in galactose fed processes. This seemed to be a result of variable galactose consumption throughout the process. Several things could account for the changes in galactose consumption rates. Induction of artemisinic acid production seemed to affect growth rate and carbon consumption. The addition of methionine reduced metabolic flow to sterol biosynthesis and diverted it to artemisinic acid biosynthesis. Sterols are an important part of yeast cell walls[7] and reduction of sterol biosynthesis would impact cell growth. Finally, the seed culture and initial fermentation process relied on glucose to accrue biomass. Cell growth lagged while transitioning from glucose consumption to galactose consumption. From a process control standpoint, a feed delivery strategy responsive to culture conditions was desirable. DO-stat algorithms can be used to deliver carbon to bioreactors as it is consumed[8] and seemed like an appropriate approach for process control for galactose induction.

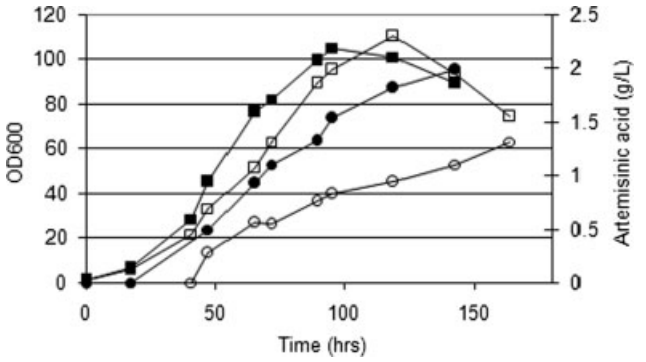

Figure 3. The effect of methionine on artemisinic acid production by *S. cerevisiae* Y51 in a galactose fed-batch process.

OD_{600} with methionine (■), artemisinic acid with methionine (●), OD_{600} without methionine (□), artemisinic acid without methionine (○).

Many DO-stat processes rely on a preprogrammed agitation and/or oxygen delivery profile. A process where oxygen delivery and feed rates were controlled simultaneously is described by Lim et al.[9] In their control strategy, when dissolved oxygen drifted above the set point, the feed rate was increased and the oxygen flow rate was decreased simultaneously. When the dissolved oxygen dropped below the set point, the oxygen flow rate was increased and the feed rate was decreased simultaneously. A similar approach was implemented in this work, where a DO-stat algorithm was developed that controlled both agitation and feeding but only one variable was changed at a time. The schematic for this control strategy is shown in Figure 4.

The control algorithm started with a growth phase that recognized the decrease in DO as the batched glucose and galactose were consumed and engaged a stirring cascade to keep the DO above 40%. Once carbon was depleted and the DO rapidly increased, the galactose feed pump was activated

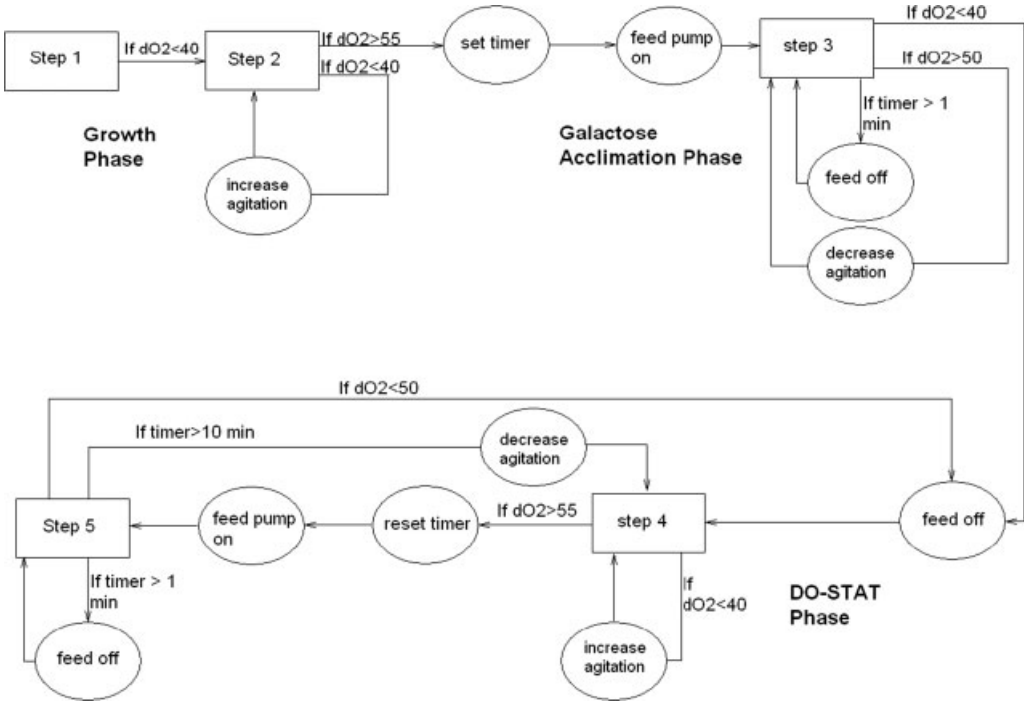

Figure 4. Control strategy for maintaining dissolved oxygen concentration and delivering galactose feed.

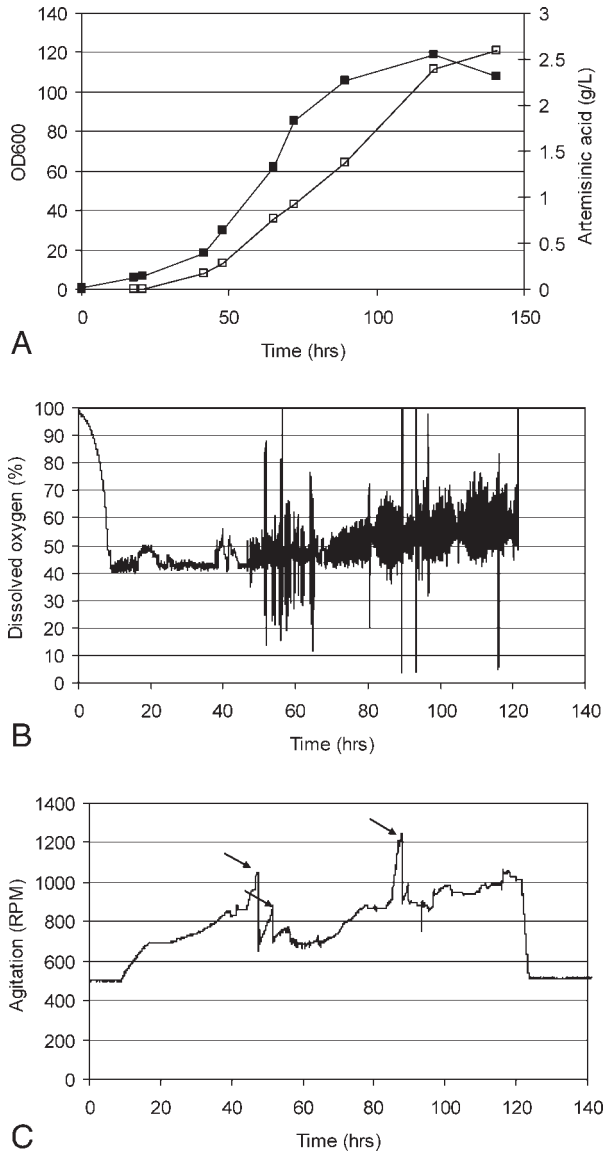

Figure 5. A. Cell growth and artemisinic acid production by *S. cerevisiae* Y51 in a DO-stat galactose fed process. OD_{600} (■), artemisinic acid (□). B. Percent dissolved oxygen in a galactose DO-stat fermentation. C. Agitation profile for a galactose DO-stat fermentation.

Arrows indicate manual adjustment to oxygen enrichment during the fermentation.

and the galactose acclimation phase of the algorithm was initiated.

The galactose acclimation phase was required for processes that started with glucose only (either as fed-batch glucose processes followed by galactose induction, or galactose fed processes where galactose was not present in the starting medium). The change from glucose to galactose consumption caused a reduction in cell growth. The galactose acclimation phase included a timer which inactivated the pump and reduced the agitation until the DO was below 50%. Once the culture adapted to galactose and began consuming it faster, the DO would again decrease (to below 40%) and the DO-stat phase of the control algorithm engaged. In this final control phase, the software increased agitation when the DO was below 40%. It also turned on the feed pump and set a timer when the DO was greater than 55%. When the timer reached 1 min, the pump turned off. If the DO dropped in response to the feed pump (below 50%), the cycle was repeated. If the DO did not drop in response to the feed pump (10 min were allowed for a response), the agitation was reduced and the control remained static until the next DO increase above 55% or decrease below 40%.

For all phases of the algorithm, the pump delivered feed at a fixed rate of 5.2 mL/min and whenever the pump engaged, it ran for 1 min. The computer checked the DO measurement every 30 s to determine if it was above or below control thresholds. The set points for the algorithm (timer intervals, DO thresholds, and pump on times) were determined empirically. A preliminary run was set up and the process behavior monitored during key process phases. Adjustments were made to the algorithm particularly during the transition from glucose to galactose feeds and during the end of the run where carbon demand slowed. The final values were selected for their robust control of carbon accumulation and feed delivery.

A galactose fed process was run with the feed delivered via the DO-stat algorithm. The initial batch medium contained 10 g/L glucose and 25 g/L galactose. The batch glucose and galactose were consumed at 39 h, at which point, the dissolved oxygen increased, initiating the feed. Since galactose was present in the initial batch medium, cells were acclimatized to galactose prior to carbon restriction and the cell response to galactose was reduction in DO to below 40%. This essentially bypassed step 3 (galactose acclimation phase) and initiated step 4. From 39 h until the process finished, the DO-stat phase (Steps 4 and 5, Figure 4) controlled feed and agitation. Maximum cell density was 120 OD_{600} with the DO-stat process (Figure 5A). Artemisinic acid production was improved by DO-stat control from 1.8 g/L up to 2.5 g/L (Figure 5A). The dissolved oxygen trace for that process is shown in Figure 5B, and agitation is shown in Figure 5C. Oxygen enrichment was required to support the cell densities achieved, and the control strategy was able to reestablish agitation and feed rates after manual adjustments to oxygen enrichment.

Use of the modified DO-stat feed control algorithm for testing high-density induction in a two-carbon process

The induced process with galactose as the primary carbon source only reached 120 OD_{600} (Figure 5A) as compared 250 OD_{600} in the uninduced glucose fed process (Figure 1). Fermentation titers can be improved by increasing culture density as long as the specific productivity of the culture is maintained. Fieschko et al.[10] used a galactose inducible promoter for the production of recombinant human immune interferon in *S. cerevisiae*. They used a glucose fed-batch process to obtain high cell density and either induced with pulses of galactose or by switching the feed to galactose only. Production was >50% higher in the process where a glucose feed was replaced with a galactose feed at induction compared to induction with galactose pulses.[10]

The galactose feed algorithm developed here allowed implementation of a glucose fed-batch process followed by galactose induction. The bioreactor was programmed with an exponential glucose feed with a doubling time of 6 h. Growth on glucose allowed the culture to grow to an OD_{600} of 150. At that time (52 h after inoculation), the feed was switched to galactose. The feed algorithm in Figure 4 was initiated. Cell growth was slower after the switch to galactose (Figure 6A); however, the culture reached a final

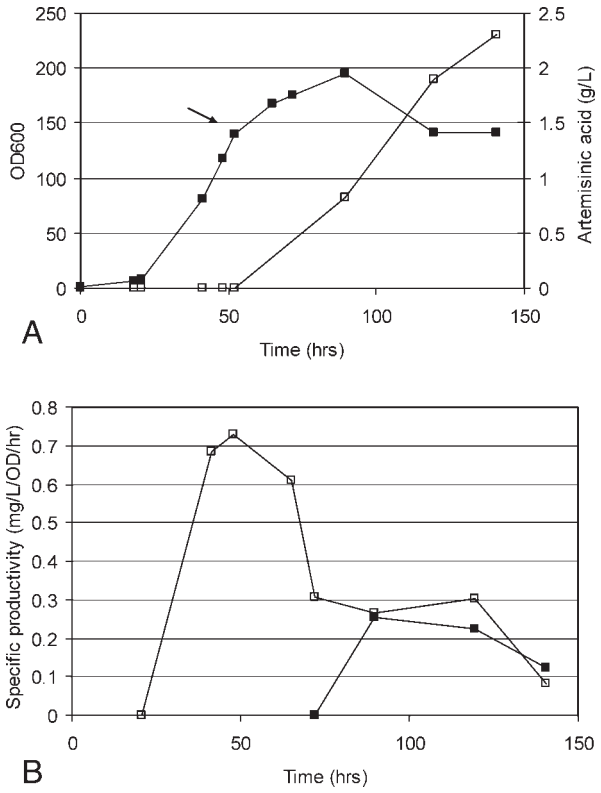

Figure 6. A. Growth and artemisinic acid production in a process where *S. cerevisiae* Y51 was grown to ~150 OD_{600} with an exponential glucose feed then induced with a galactose feed. Arrow indicates change in feed from glucose to galactose. OD_{600} (■), artemisinic acid (□). B. Specific productivity for galactose DO-stat process (Figure 5A, (□)) and glucose fed process with galactose induction (Figure 6A, (■)).

density of 200 OD_{600}, closer to that achieved by the glucose process (Figure 1). The transition from the glucose feed to the galactose feed was well controlled with the modified DO-stat algorithm. Artemisinic acid titers reached 2.3 g/L, slightly lower than the galactose fed process. The peak specific productivity for the galactose fed process was much higher than the process where the cells were grown on glucose then switched to galactose (Figure 6B). The metabolic adjustments involved in changing carbon sources may lead to reduction either in recombinant production of pathway enzymes or reduced carbon flow through the pathway once the enzymes are in place. Alternatively, the high specific productivity may be related to rapid growth. The specific productivity in the galactose process was highest from 40 to 65 h where growth was also most rapid. As growth slowed, the specific productivity decreased to a similar level as that of the glucose process induced by galactose (Figure 6B).

The galactose fed-batch process controlled by the DO-stat algorithm had the highest titer (Figure 5A) and the highest specific productivity (Figure 6B). The process was repeated two additional times. The algorithm performance was reproducible, with dissolved oxygen and stirring control comparable to that seen in Figures 5B,C. The average titer of the three runs was 2.5 g/L + 0.1.

Conclusions

A modified DO-stat algorithm was developed that can control agitation and feed rates simultaneously. With this algorithm, the oxygen-transfer rates (controlled by agitation) and feed rates responded to changes in culture conditions simultaneously. Feed delivery was well controlled throughout the process with this algorithm. Feed frequency and agitation increased with cell growth and decreased at the end of the run as metabolism slowed. This control strategy allowed for rapid process implementation and is a valuable tool for fermentation processes where growth rates and carbon consumption rates are variable, especially in cases like this where multiple carbon sources were required. DO-stat algorithms with manually set agitation or airflow require time-consuming empirical optimization and usually suffer from poor control toward the end of processes.

Heterologous production of artemisinin precursors by fermentation is an urgent area of active research interest, to ensure a consistent no-season supply of artemisinin for artemisinin-based combination therapies, the current World Health Organization recommended treatment for malaria. The strain and processes described here still require some costly inputs, including uracil, lysine, and high concentrations of galactose. However, the production cost could be brought down further in this fermentation process by repairing the biosynthetic genes for uracil and lysine production. Galactose catabolism could be knocked out in the strain, reducing the quantity of galactose required for induction. The process could be run with glucose as a sole carbon source, with the concentrations maintained below the level that inhibits induction of the GAL1 and GAL10 promoters.

Production of amorpha-4,11-diene, the isoprenoid precursor of artemisinic acid has reached 0.5 g/L in *Escherichia coli*[11] and 150–600 mg/L in *S. cerevisiae*.[12,13] Production of artemisinic acid in *S. cerevisiae* has been reported at 100 mg/L.[4] By developing a high-density fed-batch fermentation process with a DO-stat algorithm that controlled carbon delivery and agitation simultaneously, artemisinic acid production was improved 25-fold over previously reported titers.

Acknowledgments

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