# Enhanced Biosurfactant Production by *Corynebacterium alkanolyticum* ATCC 21511 Using Self-Cycling Fermentation

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**ABSTRACT:** Enhanced biosurfactant production by *Corynebacterium alkanolyticum* ATCC 21511 was accomplished in a self-cycling fermenter (SCF) on a hexadecane substrate. The phospholipid biosurfactant produced during each cycle could be monitored rapidly using fluorescence spectroscopy. By optimizing the cycling pattern of the SCF, significantly better yields of biosurfactant were obtained than previously reported for this microorganism. It was also possible to virtually eliminate the hydrocarbon residue in the product. Harvest concentrations of 1.9 g $L^{-1}$ were obtained by using a two-stage fermentation. The first step was the growth of *C. alkanolyticum* in an SCF to yield a harvest of synchronous cells. These cells were transferred to a second vessel for the production stage. The concentration of biosurfactant could be further increased to 2.7 g $L^{-1}$ by the addition of more hexadecane at the beginning of the second stage.

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**KEY WORDS:** Biosurfactant production, hydrocarbon substrate, self-cycling fermentation.

The recent interest in industrial applications of biosurfactants has been driven by the biodegradability and broad applicability of these microbial surface-active compounds (1–5). The wide range of chemical structures and their effectiveness over a broad range of temperature and pH make biosurfactants appropriate for use in fields as diverse as oil-spill remediation, food processing, and cosmetics.

A key factor hindering the widespread use of biosurfactants is that microorganisms generally produce these compounds in very small quantities. Although there are a few examples of large yields of sophorolipids (6,7), the concentrations of most biosurfactants are so low (8–13) that the recovery of these biosurfactants is both difficult and expensive (2,4,5,14,15).

Previous work with *Corynebacterium alkanolyticum* showed that this organism produces a phospholipid biosurfactant in yields of 0.5 to 0.7 g $L^{-1}$ (13). In this study, the same microorganism was grown in a self-cycling fermenter (SCF). The SCF has demonstrated enhanced yields for several biological products. The SCF technique has been described previously (16–19), but because it has several unusual features, it is useful to summarize the main features of its operation.

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Self-cycling fermentation is a computer-controlled series of batch growth cycles. A parameter related to growth, such as the concentration of dissolved oxygen (DO) in the growth medium, is monitored constantly. As growth proceeds, the DO gradually decreases in response to the demand from the cells. At some point, the limiting nutrient is completely consumed and there is a sharp decrease in growth rate. This results in an immediate increase in the DO as the cells are no longer removing oxygen as quickly as it is being added to the medium. The computer monitoring the DO recognizes the resulting minimum and starts a series of procedures with the result that half of the working volume in the fermenter is harvested and then this volume is replaced with an equal amount of fresh medium. With the replenished nutrients, growth immediately resumes, the DO starts to decrease, and the cycle is repeated. This technique usually results in synchronous growth of the microbes involved after several cycles have been completed.

SCF is flexible and can be modified by adding a period of delay after the minimum is detected. This extended cycle is particularly useful for the production of secondary metabolites such as biosurfactants. Whatever mode of operation is being used, SCF is a quick method of performing many highly reproducible batch fermentations. The objective of this work was to use the SCF both to maximize the final concentration of a biosurfactant and to minimize the concentration of hydrocarbon residue.

## EXPERIMENTAL PROCEDURES

*Microorganism and culture conditions.* The organism used for this study was *C. alkanolyticum* ATCC 21511. Cultures were maintained on nutrient agar (Difco) and were transferred regularly. Stock cultures were maintained at −70°C. The medium used for fermentations in the SCF was based on that of Nakao *et al.* (13) and had the following composition: $(NH_4)_2SO_4$, 0.6% (wt/vol); $NH_4NO_3$, 0.3%; $K_2HPO_4$, 0.25%; $KH_2PO_4$, 0.10%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.005%; $MnSO_4 \cdot 7H_2O$, 0.002%; $ZnSO_4 \cdot H_2O$, 0.001%; and $CoCl_2 \cdot 6H_2O$, 0.001%.

Hexadecane was used as the carbon source in all fermentations. Hexadecane is a common hydrocarbon substrate because it is a relatively inexpensive pure hydrocarbon. It was not necessary to sterilize the hexadecane.

The setup used for the self-cycling fermentations was a cyclone fermenter. Both the fermenter and the SCF technique

have been described in detail previously (16–19). In the present work, an online measurement of DO was fed back to the computer, which controlled the cycling of the fermenter. The cycling could be initiated just after the minimum in the DO trace was observed or after a time delay. This longer mode of operation was referred to as an extended cycle. In the work presented here, the time delay was usually 90 min. Aeration (sterile air) was 120 mL $min^{-1}$. The temperature of the reactor was maintained at 27°C. In most experiments, 1.1 mL of hexadecane was added to the fermentation broth at the beginning of each cycle using a syringe pump. The cyclone fermenter is particularly suitable for growth on an insoluble substrate such as hexadecane because it ensures that the hydrocarbon is well dispersed throughout the total working volume (1 L).

The SCF technique generates two types of data. Data collected by analyzing the harvest sample at the end of each cycle are termed end-of-cycle data. It is also possible to collect samples throughout a particular fermentation cycle, and these lead to intracycle data. Both of these types of data can be collected either for a truncated cycle, which is harvested just after the DO reaches a minimum, or for an extended cycle, which is harvested at a fixed period of time after the DO reaches a minimum.

Data such as those in Table 1 are average values with confidence intervals (95%) for biosurfactant and residual hydrocarbon concentrations and cycle time for different modes of SCF operation. Both the mean values and the confidence intervals were determined with data collected from a minimum of three groups of consecutive cycles. Each of these sets of cycles contained at least eight cycles. The confidence intervals for the listed measurements demonstrate that the SCF technique leads to end-of-cycle data that are very repeatable. As well, the patterns observed for the DO traces were highly reproducible for experiments done using the same growth conditions. In the figures only a few cycles are shown but these were taken from a larger series of stable cycles.

For the experiments in which hexadecane was added continuously over the duration of a cycle, hexadecane was kept in a sterile glass reservoir and circulated with the aid of a peristaltic pump (Masterflex; Cole Parmer Instrument Co., Vernon Hills, IL). Tubing outside the reactor was Masterflex Tygon® fuel and lubricant tubing (6401-13) with 0.8 mm i.d. Within the fermenter, Masterflex platinum-cured silicon tubing with 0.8 mm i.d. was used. One hundred holes were pierced in this tubing using a metal pin (diameter, 0.42 mm). Tubing entering or exiting the reactor was attached to 20-gauge cannulae that pierced septa in the wall of the reactor. With this reactor, hexadecane was added at a rate of 0.114 mL $L^{-1}$ $h^{-1}$.

For the two-stage mode of operation 100-mL samples were withdrawn from the fermenter upon detection of the minimum in DO. These samples were placed in 500-mL Erlenmeyer flasks and incubated at the same temperature as that of the fermenter (New Brunswick Scientific Model G25 Incubator Shaker). Between 0 and 0.9 g of hexadecane were added to the flasks at the beginning of the second stage. The flasks were monitored for biosurfactant and hydrocarbon concentration.

*Biomass and cell counts.* End-of-cycle biomass measurements were made using a standard dry weight analysis (20) modified to remove excess hexadecane. Triplicate 20-mL samples were centrifuged at $8000 \times g$ for 20 min at 4°C, and residual hexadecane solidified on the surface of the aqueous phase. The hexadecane was removed as described previously (21). Final biomass concentrations were reported as g dry cell mass per L fermentation broth.

A standard colony count method, based on that outlined by Koch (20), was used, in triplicate, to determine the number of viable cells in the fermenter. A 1-mL sample of broth was placed in an autoclaved Nalgene bottle, diluted to a final volume of 100 mL with sterilized, distilled water, and sealed. After 50 inversions, 1 mL of the solution was placed in a second bottle and diluted to 100 mL with sterilized, distilled water. This solution was also shaken 50 times. The final dilution consisted of 1 mL of sample from the second dilution and 9 mL of sterile water. A 0.1 mL sample was taken from the final dilution bottle, spread on nutrient agar plates, and allowed to grow in an incubator for 36 to 48 h at 27°C. The number of cells in the fermenter was calculated using the number of cell colonies grown on the plate and the total dilution factor used.

*Hexadecane measurement.* Hexadecane concentrations were measured based on an earlier procedure (16). Samples (2.0 mL) were taken from the fermenter, and 10.0 µL aliquots of pentadecane (internal standard) were added. The solution was mixed vigorously (Vortex Genie; Scientific Industries, Bohemia, NY) for 1 min. A 5.0 mL volume of isooctane was added to the sample, and the solution was again mixed for 1 min. Samples of the hydrocarbon phase were analyzed in a gas chromatograph with an FID and integrator. The column was Supelco SPB-5 (Belle-

**TABLE 1**
**Concentrations of Biosurfactant and Residual Hexadecane in End-of-Cycle Harvest Samples for Different Modes of Fermentation**

| Fermentation mode | Cycle time (min) | Time after minimum DO (h) | Final biosurfactant[a] (g $L^{-1}$) | Hexadecane residue[a] (g $L^{-1}$) |
|---|---|---|---|---|
| Continuous hexadecane addition | 120 | 0 | 0 | 0.02 |
| Series of extended cycles | 220 ± 15 | 1.5 | 0.75 ± 0.06 | 0.14 ± 0.06 |
| Truncated cycles | 140 ± 3 | 0 | 1.10 ± 0.07 | 0.35 ± 0.06 |
| Single extended cycle (after truncated series) | 180 | 1.5 | 1.44 | 0.18 |

[a]Values are presented as mean ± 95% confidence interval, based on a minimum of three groups of consecutive cycles. DO, dissolved oxygen.

fonte, PA) and the carrier gas (helium) flow rate was 5 mL/min. The initial column temperature was 65°C; this was maintained for 2.5 min before being increased at a rate of 10°C $min^{-1}$ until reaching a final temperature of 200°C.

*Surface tension measurement.* Surface tension measurements were made using the Model 215 Autotensiomat surface tension analyzer (Fisher, Toronto, Ontario, Canada), which employed the du Nouy method as described elsewhere (8,12,21,22).

*Phospholipid analysis.* Lipids were extracted from the fermentation broth using a chloroform/methanol solvent mixture (2:1 vol/vol) and potassium chloride according to the method of Folch *et al.* (23). The phospholipids were removed from the total lipid extract using acetone precipitation according to the method of Kates (24). The solid phospholipids were dried in a vacuum desiccator over potassium hydroxide. TLC was done using the solvent mixture chloroform/methanol/acetic acid/water (40:25:3:7, by vol).

Fluorescence spectroscopy was used to determine the concentration of the phospholipid biosurfactant in the samples. A Shimadzu (Kyoto, Japan) RF-1501 spectrofluorophotometer with a xenon arc light bulb was used. Samples taken from the fermenter were centrifuged to remove biomass and then diluted 100-fold using distilled water in order to obtain readings that fell within the scale of the fluorometer.

## RESULTS AND DISCUSSION

*Fluorescence spectroscopy to monitor biosurfactant concentrations.* Previously, an indirect method has been used to quantify the amount of a biosurfactant present in a fermentation broth, such as the surface tension or the dilution required to reduce the concentration below the critical micelle concentration (2,4,12,15,22,25). This is an inaccurate method, and it takes a significant amount of time to carry out the required serial dilutions. The self-cycling fermentations are relatively short, and in order to follow the production of biosurfactants, it was necessary to develop a quick and accurate technique for measuring their concentration.

It was found that the phospholipid biosurfactant produced by *C. alkanolyticum* could be measured by taking advantage of its fluorescence spectrum. Samples of the broth, after removing the biomass, exhibited fluorescence spectra with a peak at 445 nm when the excitation wavelength was 283 nm. The separation procedure used to concentrate the active component is outlined in the Experimental Procedures section. The intermediate products obtained from each step of this procedure were all analyzed for both surface activity and fluorescence properties. All of these isolates, and the final product, lowered the surface tension to below 32 mN $m^{-1}$ and had a fluorescence peak at 445 nm when excited at 283 nm. There was no interference, and the adsorption was linear with concentration. The final product was a single spot on TLC. This material was used to prepare a fluorescence standard curve (not shown), which was used to monitor the concentration of the surface-active phospholipid in subsequent work. To confirm the association between fluorescence and concentration of the surface-active agent, this concentration was periodically determined using the more traditional method of dilution to the CMC.

*Improving the production of the biosurfactant.* Previous research with the SCF technique has demonstrated that it can result in an enhanced yield of certain microbial metabolites (18,19). This work was undertaken to test if SCF could be used to improve the concentration and quality of a biosurfactant.

Previous work with *C. alkanolyticum* typically produced 0.52 g $L^{-1}$ of phospholipid biosurfactant (13). This was obtained with very large excesses of hexadecane [10% (wt/vol)], and only 4% of this product was extracellular; the remainder had to be extracted from the cells. Use of an agent such as penicillin to increase the permeability of the cell walls showed a slightly increased concentration of phospholipid to 0.69 g $L^{-1}$ with 60% of this being extracellular. Since the goal of the current work was to minimize separation costs, only the extracellular concentrations of the biosurfactant are reported here.

In the following discussion, comparisons are made based on the final concentration of biosurfactant in the broth. This is because, owing to the small concentrations involved, the most significant cost for the large-scale production of biosurfactants is the cost of separation of the product (2,4,5,14,15). Another separation problem for biosurfactant production is the residue of unutilized hydrocarbon substrate that is left after the fermentation is completed. This problem is often ignored, but there are several papers in which it is mentioned (4,10–12,26). The extraction techniques used to collect the crude product also extract the hydrocarbon residue, which remains as a contaminant unless extra steps are taken to remove it.

It has been proposed that microorganisms produce biosurfactants to facilitate the uptake of immiscible hydrocarbon substrates (3,9,27–30). It has been stated that the presence of a hydrocarbon substrate will actually induce the production of a biosurfactant (4). This might lead to a solution of both the foregoing problems by limiting the amount of hydrocarbon available during the fermentation. If these effects are important for the production of phospholipid from *C. alkanolyticum*, the constant, trace amount of carbon source might result in an enhanced yield of biosurfactants as well as control the amount of hydrocarbon residue.

Experiments were carried out in which the hydrocarbon was added continuously throughout the cycle in such a way that the total amount of hydrocarbon present at any time was barely detectable. The average results for all of the cycles using this continuous addition are in Table 1. The positive result from this experiment was that there was no hydrocarbon residue. Unfortunately, neither was there any product and this approach was abandoned.

The remaining experiments were all performed with the addition of 0.85 g of the hydrocarbon to the 1-L reactor at the beginning of each cycle. Figure 1 shows an example of the change in DO during several cycles in an SCF of *C. alkanolyticum* after the system reached a stable pattern. In these experiments, the control pattern was an extended mode (18), with the fermenter being cycled 90 min after the DO recorded a min-

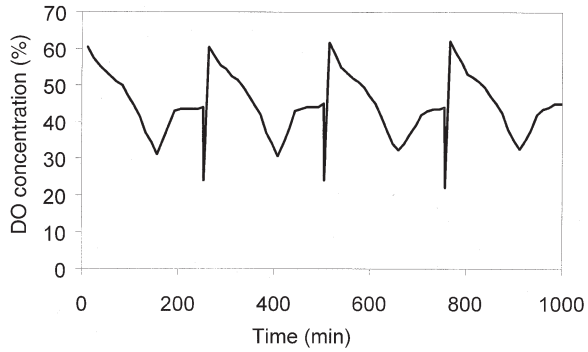


**FIG. 1.** Dissolved oxygen (DO) profile, in % saturation, throughout four consecutives cycles of *Corynebacterium alkanolyticum* growing on 0.11% hexadecane. In each of these extended cycles, harvesting was delayed for 90 min after the minimum dissolved oxygen concentration.

imum value. The length of each batch was extended in an attempt to ensure that all of the hydrocarbon had been metabolized, but it can be seen in Table 1 that there was still a small amount (0.14 g $L^{-1}$) of hydrocarbon in the product. The final concentration of product, 0.75 g $L^{-1}$, was acceptable. The value reported previously for similar conditions was 0.52 g $L^{-1}$.

It is also possible to follow the amount of product present within each of the cycles. Figure 2 shows the typical intracycle pattern for the extended mode. It can be seen that the maximal concentration of product is much earlier in the cycle than the point of harvest. This type of production pattern has been reported for other fermentations in which there is a maximum in biosurfactant concentration followed by a significant decrease by the end of the fermentation as the biosurfactants are first synthesized and then metabolized by the microbes (8,12,13,21,25,27).

It would be desirable to harvest the product at the point of maximal concentration. Unfortunately, as can be seen in Figure 2, this is well before the minimum DO. For a typical SCF cycle, the minimum DO is the point at which the limiting nutrient has run out and growth stops. The cells in an SCF cycled at the point of the minimum DO tend to become synchronized and most of the cells double within a very short period of time, which usually occurs just before the minimum DO (17–19).

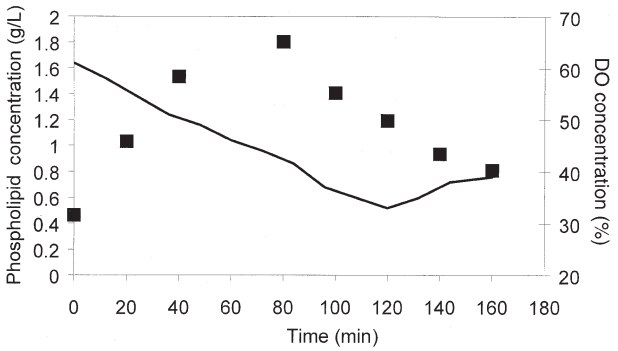


**FIG. 2.** Intracycle DO (—) and phospholipid (■) concentrations during a single extended cycle extended for 90 min after the minimum DO. This cycle followed a series of extended cycles. For abbreviation see Figure 1.

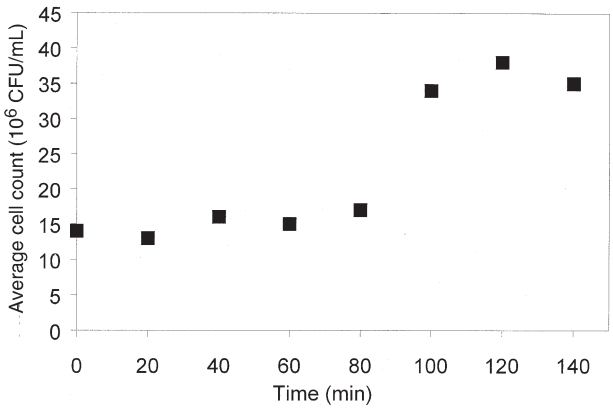


**FIG. 3.** Average viable cell counts for samples taken during a single truncated self-cycling fermenter cycle after a series of truncated cycles. The minimum DO concentration for this cycle occurred at 115 min. The number of cells doubled between 80 and 100 min. This synchronous behavior was observed only for cycles that followed a series of stable cycles, all harvested just after the minimum in DO was observed. CFU, colony-forming units; for other abbreviation see Figure 1.

This behavior is observed for this system, as can be seen in Figure 3 for one SCF cycle in a series of cycles that were truncated at the point of minimal DO. If this type of SCF cycle is harvested before the minimum DO, the culture is washed out, as the cycle period is shorter than the doubling time (16–19).

A compromise was to harvest the SCF at the point of minimum DO at which the concentration of product should still be higher than at the end of the extended period. The first three cycles in Figure 4 are typical of the pattern observed for these truncated cycles. The final concentration did improve to 1.1 g $L^{-1}$ (Table 1), but there was also a large increase in the amount of residual hexadecane (0.35 g $L^{-1}$). Looking at a single truncated cycle (not shown), one can see that there had been a change in the pattern of biosurfactant production when compared to the typical extended cycle (Fig. 2). The point of maximal biosurfactant concentration (1.5 g $L^{-1}$) now came later in the cycle (150 min), at the point at which the DO reached a minimum and the cycle was harvested. This leads to the question of whether the concentration would have con-

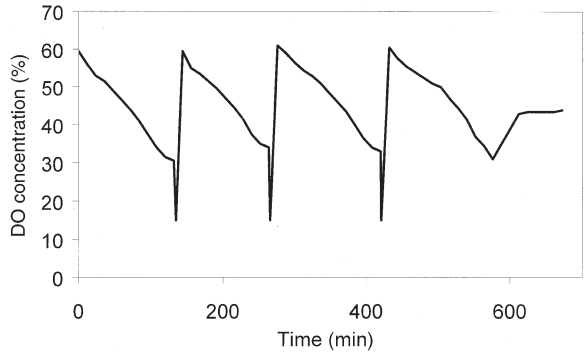


**FIG. 4.** DO profile throughout four consecutive cycles of *C. alkanolyticum* growing on 0.11% hexadecane. The first three truncated cycles were harvested at the time when the DO concentration reached a minimum. The fourth, extended cycle was not harvested until 90 min after the minimum DO concentration. For abbreviation see Figure 1.

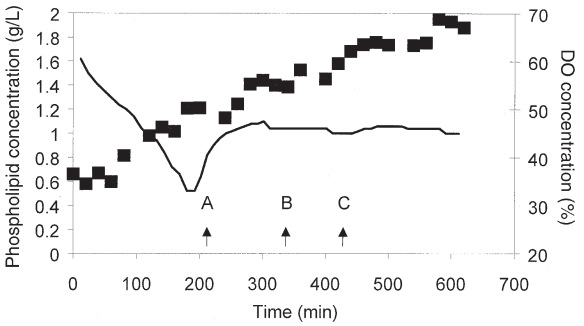


**FIG. 5.** Intracycle DO (—) and phospholipid (■) concentrations for an extended cycle. The minimum DO concentration was attained at time A. At time B, 0.18 g of hexadecane was added to the fermenter. At time C, an additional 0.34 g of hexadecane was added. For abbreviation see Figure 1.

tinued to increase in one of these truncated cycles if it had not been harvested at the minimum DO.

To answer this question, a single extended cycle was programmed after a series of truncated cycles. The fourth cycle in Figure 4 is an example of this type of experiment. In this type of experiment the biomass continued to convert hexadecane to biosurfactant even after growth had stopped. The average final concentration of biosurfactant (1.7 g $L^{-1}$) for this type of cycle was significantly higher than that observed for either the truncated cycles or a series of extended cycles, and the final concentration of hexadecane (0.18 g $L^{-1}$) was as low as that observed for a series of extended cycles (Table 1).

The experiments with cycles extended for 90 min after the minimum DO demonstrated two very different types of behavior. The single extended cycle, after a series of truncated cycles, always resulted in more product than one cycle in a series of consecutive extended cycles (e.g., Fig. 2).

There is another difference between these two types of extended cycles. As discussed above, the cells in the truncated cycles are synchronized because the number of cells doubles within a period of time much less than the period of the cycle (Fig. 3). A similar pattern was observed for cells growing in a single extended cycle following a series of truncated cycles. However, when the fermenter was run as a consecutive series of extended cycles, this synchrony was lost and the cells doubled over a wide period of time. This could mean that the higher yield is related to the cells being synchronized in one type of extended cycle but not in the other type.

**TABLE 2**
**Final Concentration of Biosurfactant and Residual Hydrocarbon for Two-Stage Experiments With or Without Additional Hexadecane Added to the Second Stage**

| Additional hexadecane (g $L^{-1}$) | Duration of second stage (h) | Hexadecane residue (g $L^{-1}$) | Final biosurfactant (g $L^{-1}$) |
|---|---|---|---|
| 0 | 24 | 0.006 | 1.89 |
| 0.15 | 20 | 0.02 | 2.29 |
| 0.3 | 20 | 0.06 | 2.25 |
| 0.45 | 20 | 0.33 | 2.74 |

If it is necessary to run a series of truncated cycles to establish synchronous growth followed by a single extended cycle with a high concentration of product, the extra time required cancels any benefit. The last experiments were attempts to obtain the advantages of the extended cycle over shorter periods of time. The first approach was to increase the amount of product in a synchronized extended cycle by adding more hexadecane after the minimum DO was observed. For example, in Figure 5, two separate additions of small amounts of hexadecane significantly increased the final concentration of biosurfactant. This method still requires a longer period of time but it does show that, once the biomass is produced, it can be used to convert hexadecane to biosurfactant.

The last type of experiment decoupled the two stages of the fermentation. The cells were produced in the SCF in a series of truncated cycles resulting in synchronous growth. When each cycle was harvested at the minimum DO, the harvest was put into a second vessel, with or without additional hexadecane, and left for an extended period of time. The results in Table 2 demonstrate that this second stage can be manipulated to optimize both the final concentration of product and the amount of hexadecane residue.

In previous studies, it has been possible to achieve higher concentrations of biosurfactants by using higher concentrations of hydrocarbon. However, this approach results in lower yields of the product relative to both substrate and biomass. This study with *C. alkanolyticum* has demonstrated that when the growth in the SCF is synchronous there is an improvement in the yield of the biosurfactant, but this requires a series of truncated cycles followed by a single extended cycle with a high yield. An extended cycle will also lead to a decrease in the amount of hexadecane residue but this overall procedure takes too much time. The most efficient approach is to combine the useful aspects of these procedures. The cells can be grown in a fermenter using a series of truncated cycles. The harvest from each of these cycles can be put in secondary vessels, and the residence time and amount of additional hexadecane added can be optimized for quantity and quality of the biosurfactant.

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## REFERENCES

1. Rosenberg, E., Microbial Surfactants, *CRC Crit. Rev. Biotechnol. 3*:109–132 (1986).
2. Cooper, D.G., Biosurfactants, *Microbiol. Sci. 3*:145–149 (1986).
3. Parra, J.L., J. Guinea, M.A. Manreso, M. Robert, M.E. Mercade, F. Comelles, and M.P. Bosch, Chemical Characterization and Physicochemical Behavior of Biosurfactants, *J. Am. Oil Chem. Soc. 66*:141–145 (1989).
4. Lin, S.C., Biosurfactants: Recent Advances, *J. Chem. Technol. Biotechnol. 66*:109–120 (1996).
5. Desai, J.D., and I.M. Banat, Microbial Production of Surfactants and Their Commercial Potential, *Microbiol. Molec. Biol. Rev. 6*:47–64 (1997).

6. Ochsner, U.A., A.K. Koch, A. Fiechter, and J. Reiser, Isolation and Characterization of a Regulatory Gene Affecting Rhamnolipid Biosurfactant Synthesis in *Pseudomonas aeruginosa, J. Bacteriol. 176*:2044–2054 (1994).
7. Stuwer, O.R., R. Hommel, D. Haferburg, and H.P. Keiber, Production of Crystalline Surface-Active Glycolipids by a Strain of *Torulopsis apicola, J. Biotechnol. 6*:259–269 (1987).
8. Cooper, D.G., J.E. Zajic, and D.F. Gerson, Production of Surface-Active Lipids by *Corynebacterium lepus, Appl. Environ. Microbiol. 37*:4–10 (1979).
9. Goswami, P., and H.D. Singh, Different Modes of Hydrocarbon Uptake by Two *Pseudomonas* Species, *Biotech. Bioeng. 37*:1–11 (1991).
10. Gutierez, J.R., and L.E. Erickson, Hydrocarbon Uptake in Hydrocarbon Fermentations, *Ibid. 19*:1331–1349 (1977).
11. Koch, A.K., O. Kappeli, A. Fiechter, and J. Reiser, Hydrocarbon Assimilation and Biosurfactant Production in *Pseudomonas aeruginosa* Mutants, *J. Bacteriol. 173*:4212–4219 (1991).
12. MacDonald, C.R., D.G. Cooper, and J.E. Zajic, Surface-Active Lipids from *Nocardia erythropolis* Grown on Hydrocarbons, *Appl. Environ. Microbiol. 41*:117–123 (1981).
13. Nakao, Y., T. Kanamaru, M. Kikuchi, and S. Yamatodani, Extracellular Accumulation of Phospholipids, UDP-*N*-Acetylhexosamine Derivatives and L-Glutamic Acid by Penicillin-Treated *Corynebacterium alkanolyticum, Agric. Biol. Chem. 37*:2399–2404 (1973).
14. Donnelly, M.J., and J.D. Bu'Lock, The Synthesis of Sucrose Esters from Telomer Monocarboxylic Acids, *J. Chem. Technol. Biotechnol. 61*:187–195 (1994).
15. Reiling, H.E., U.T. Wyass, L.H. Guerra-Santos, R. Hirt, O. Kappeli, and A. Fiechter, Pilot Plant Production of Rhamnolipid Biosurfactant by *Pseudomonas aeruginosa, Appl. Environ. Microbiol. 51*:985–989 (1986).
16. Brown, W.A., and D.G. Cooper, Self-Cycling Fermentation Applied to *Acinetobacter calcoaceticus* RAG-1, *Ibid. 57*:2901–2906 (1991).
17. Brown, W.A., and D.G. Cooper, Hydrocarbon Degradation by *Acinetobacter calcoaceticus* RAG-1 Using the Self-Cycling Fermentation Technique, *Biotechnol. Bioeng. 40*:797–805 (1992).
18. McCaffrey, W.C., and D.G. Cooper, Sophorolipids Production by *Candida bombicola* Using Self-Cycling Fermentation, *J. Ferment. Bioeng. 79*:146–151 (1995).
19. Wentworth, S.D., and D.G. Cooper, Self-Cycling Fermentation of a Citric Acid-Producing Strain of *Candida lipolytica, Ibid. 81*:400–405 (1996).
20. Koch, A.L., Growth Measurement, in *Methods for General and Molecular Bacteriology*, edited by P. Gerhardt, American Society for Microbiology, Washington, DC, 1994, pp. 249–277.
21. Neufeld, R.J., J.E. Zajic, and D.F. Gerson, Growth Characteristics and Cell Partitioning of *Acinetobacter* on Hydrocarbon Substrates, *J. Ferment. Technol. 61*:315–321 (1983).
22. Persson, A., and G. Molin, Capacity for Biosurfactant Production of Environmental Pseudomonas and Vibrionaceae Growing on Carbohydrates, *Appl. Microbiol. Biotechnol. 26*:439–442 (1987).
23. Folch, J., M. Lees, and G.H. Sloane Stanley, A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*:497–509 (1957).
24. Kates, M., *Techniques of Lipidology: Isolation, Analysis, and Identification of Lipids,* American Elsevier Publishing, New York, 1975, pp. 394–395.
25. Cooper, D.G., and D.A. Paddock, *Torulopsis petrophilum* and Surface Activity, *Appl. Environ. Microbiol. 46*:1426–1429 (1983).
26. Kennedy, R.S., W.R. Finnerty, K. Sudarsanan, and R.A. Young, Microbial Assimilation of Hydrocarbons, *Arch. Microbiol. 102*:75–83 (1975).
27. Déziel, E., G. Paquette, R. Villemur, F. Lepine, and J.-G. Bisaillon, Biosurfactant Production by a Soil *Pseudomonas* Strain Growing on Polycyclic Aromatic Hydrocarbons, *Appl. Environ. Microbiol. 62*:1908–1912 (1996).
28. Hisatsuka, K., T. Nakahara, N. Sano, and K. Yamada, Formation of Rhamnolipid by *Pseudomonas aeruginosa* and Its Function in Hydrocarbon Fermentation, *Agric. Biol. Chem. 35*:686–692 (1971).
29. Ito, S., and S. Inoue, Sophorolipids from *Torulopsis bombicola*: Possible Relation to Alkane Uptake, *Appl. Environ. Microbiol. 43*:1278–1283 (1982).
30. Zhang, Y., and R.M. Miller, Effect of Rhamnolipid (Biosurfactant) Structure on Solubilization and Biodegradation of *n*-Alkanes, *Ibid. 61*:2247–2251 (1995).

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