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Optimization of the Sistrom Culture Medium for Large-Scale Batch Cultivation of *Rhodospirillum rubrum* under Semiaerobic Conditions with Maximal Yield of Photosynthetic Membranes

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The defined medium A of W. R. Sistrom (W. R. Sistrom, J. Gen. Microbiol. 22:77–85, 1960) has been modified to allow the growth of *Rhodospirillum rubrum* in large-scale batch cultures under dark, semiaerobic conditions. The simultaneous use of two substrates, NH_4-succinate (46 mM) and fructose (0.3%), which are utilized in aerobic and fermentative metabolism, respectively, leads to very high cell densities with a maximal yield of photosynthetic membranes.

The phototrophic purple non-sulfur bacterium, *Rhodospirillum rubrum*, will grow with a wide variety of carbon and energy sources under aerobic, anaerobic, or fermentative conditions (1, 5–7, 9–11). In recent years, mutants which have lost some component of the photosynthetic apparatus but which are still able to form the specialized intracytoplasmic membranes containing the photosynthetic apparatus have become an indispensable tool for the study of structure-function relationships (4). Usually, the induction of photosynthetic membranes is routinely performed by shaking aerobic cultures slowly in the dark over a period of 2 to 4 days. The low aeration level of the cultures causes the local partial O_2 pressure to fall below a critical value (approximately 5%) (1), whereupon gene expression leading to the accumulation of gene products involved in photosynthetic light harvesting is initiated. In such experiments, a 1-liter culture volume will yield quantities of intracytoplasmic membranes after cell breakage sufficient for subsequent biochemical analysis, and this technique is widely employed.

However, for the larger culture volumes (e.g., 20 liters and above) necessary for isolating large quantities of selected membrane proteins for the purpose of crystallization, we have found it extremely difficult to adapt the commonly used media (3, 8) for large-scale batch cultures. Typically, exhaustion of the substrate concomitant with a dramatic rise in pH, overproduction of cytochromes, and a low yield of photosynthetic membranes are observed.

Although the above-mentioned problems might be solved by the application of continuous culture techniques, these require specialized expertise and equipment, which are often not available. For a slowly growing organism such as *R. rubrum*, maintenance of sterile conditions is also more difficult for continuous culture than for batch cultures. For these reasons, we have chosen to optimize the medium to maximize the yield of bacteriochlorophyll (BChl) but to avoid the major difficulties of early substrate exhaustion accompanied by a rise in pH. It has been shown previously that for *R. rubrum*, the concentration of BChl in vivo, measured here at 880 nm, is directly proportional to the amount of photosynthetic membranes present per cell (2). In this paper, the terms BChl and photosynthetic membranes will therefore be used interchangeably to describe or quantify the formation of the photosynthetic apparatus.

Growth of bacteria and strains. The wild-type strain, *R. rubrum* S1, was obtained from the German Collection of Microorganisms (Braunschweig, Germany). *R. rubrum* ST2, a carotenoidless mutant, was produced by Tn*5* mutagenesis of the wild-type strain and will be described in detail elsewhere. The precultures used for an inoculum were initially grown from a single colony without (*R. rubrum* S1) or with (*R. rubrum* ST2) kanamycin selection (20 μg/ml). Unless otherwise stated, growth curves from *R. rubrum* S1 were obtained at 30°C with 100-ml shake flasks with four baffles, which were shaken in the dark at 120 rpm with a 4-cm throw. A 5-ml volume of a preculture, grown anaerobically unless otherwise stated, was used as an inoculum.

Optical density at 660 and 880 nm was measured with a Kontron 810 spectrophotometer with a 4-mm-path-length cuvette. We did not make a correction for the turbidity at 880 nm as described by Holt and Marr (2), as absolute values for BChl were not required. The number of cells per ml was calculated by direct serial dilution plating and by the following conversion factor: an A_{660} (1 cm) of 1.4 = 5×10^8 cells per ml (5a). For these small-scale batch cultures, the pH was not under external control.

Large-scale batch cultures (20 liters) were grown in the dark at 30°C with a 42-liter MBR (Chemap) bioreactor. If necessary, the pH of the medium was maintained at pH 6.8 by using 4 N HCl, and the partial O_2 pressure was monitored with a built-in O_2 electrode. The input of air was regulated mechanically by a microprocessor-controlled valve, and the culture was stirred at 100 rpm throughout. In most cases, the mutant *R. rubrum* ST2 was substituted for the wild-type strain for two reasons. (i) The absence of the carotenoid absorption facilitated the spectral monitoring of cytochrome production, and (ii) the kanamycin selection simplified the problem of maintaining sterile conditions. Control experiments with *R. rubrum* S1 in 20-liter batches showed the growth rates of the two strains to be comparable under aerobic, chemoheterotrophic conditions.

The classical Sistrom medium (8) used initially and its modification employed in this study are compared in Table 1.

Figure 1a shows a typical growth curve obtained from *R.*

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TABLE 1. Comparison of Sistrom and M2SF medium

Substance	Concn (mM) [%] in:	
	Sistrom medium	M2SF
Phosphate buffer	23	46
Na-succinate	20	
NH_4Cl	8	
NH_4-succinate		40
HEPES		50
Fructose		18 [0.3]

rubrum S1 in a semiaerobic 50-ml batch culture with Sistrom medium containing sodium succinate (20 mM) as the sole carbon source. The generation time under these conditions is approximately 7 h, and the stationary phase is reached at approximately 40 h, at which time the pH has risen from an initial value of 6.8 to pH 8.3. After 40 h, a drop in the A_{660} is observed, partly due to cell lysis. In a large-scale batch culture (20 liters) with a 2-liter inoculum, the growth characteristics are broadly similar but the magnitude of the pH change concomitant with the exhaustion of the substrate is much more severe. Typically, subsequent to culture inoculation (5 to 10% inoculum) in Sistrom medium, the cells rapidly consumed large quantities of O_2 within 12 h by aerobic metabolism of succinate, thus causing the partial O_2 pressure to fall to approximately 5% (of air-saturated medium), whereupon BChl synthesis was induced. The consumption of succinate was accompanied by a rapid rise in pH, requiring large quantities of HCl for correction. After 24 h, the substrate was nearly exhausted and growth slowed. At this point, for reasons which are still unclear, the cells repressed BChl synthesis and produced large amounts of cytochromes, resulting in a brownish culture. Further addition of substrate caused growth to resume but only to a limited extent, presumably because of the accumulation of salt arising from the neutralization of the medium with acid.

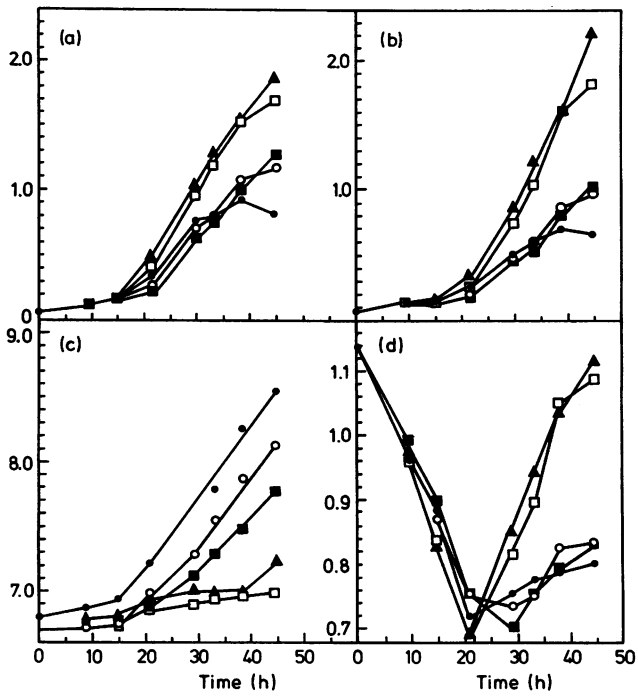

FIG. 1. Growth profiles obtained from 50-ml batch cultures of *R. rubrum* S1. Panels: a, A_{660}; b, A_{880}; c, pH; d, A_{880}/A_{660}. Symbols represent the following modifications of Sistrom medium: ●, no modification; ○, 40 mM phosphate buffer–46 mM NH_4-succinate; ■, 40 mM phosphate buffer–46 mM NH_4-succinate–50 mM HEPES; □, 40 mM phosphate–46 mM NH_4-succinate–50 mM HEPES–0.3% fructose; ▲, 40 mM phosphate–46 mM NH_4-succinate–0.3% fructose, no HEPES. Optical density measurements were performed with a 4-mm-path-length cuvette. One-milliliter culture aliquots were taken at various times and measured immediately.

Variation of the substrate counterion and concentration dependence. Although initial calculations suggested that, in Sistrom medium, ammonia may be limiting as a nitrogen source, no change in the growth characteristics was observed when the ammonia concentration was increased by up to fivefold (40 mM) under either aerobic or anaerobic conditions. Neutralization of the succinic acid with NH_4OH (final [NH_4^+], 20 mM), by the rationale that the uptake of both components should reduce the rise in pH of the medium with substrate consumption, led to a small decrease in pH compared with that observed for Sistrom medium. However, raising the [NH_4-succinate] 2-fold led to a 1.5-fold increase in cell density (Fig. 1a), with almost no effect upon the amount of BChl produced per cell (Fig. 1b and d). Further increases in the substrate concentration were inhibitory for growth.

Variation of the buffer. In Sistrom medium, the phosphate buffer concentration is 23 mM. Raising the buffer concentration to 46 mM decreased the pH at 40 h from pH 8.3 (Sistrom) to pH 7.9 (Fig. 1c), with no effect upon cell growth. Higher concentrations proved strongly inhibitory for growth, possibly by precipitating essential elements, e.g., iron. The addition of 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) to the growth medium caused a dramatic lowering of the pH at 40 h to pH 7.4. In addition, the phosphate-HEPES buffer allowed growth to continue for up to 50 h before the stationary phase was reached (pH 7.8) (Fig. 1a). Higher concentrations of HEPES were inhibitory for cell growth.

Succinate-fructose as a two-substrate carbon source. The 20 mM phosphate–40 mM HEPES–40 mM NH_4-succinate formulation described above proved to be difficult to adapt to a 20-liter batch culture. Although the rise in pH due to substrate exhaustion was dramatically decreased (only 25% of the amount of HCl used to correct Sistrom medium), the substrate, succinate, was rapidly consumed within the first 24 h, after which time cytochrome production increased and the yield of chromatophores was very low. Attempts to adjust the O_2 tension to limit substrate consumption or to continuously add fresh substrate proved to be unsuccessful for reproducibly obtaining reasonable yields of chromatophores.

For this reason, we turned to a two-substrate medium, containing both NH_4-succinate (40 mM) and fructose (0.3%, 18 mM) (7). Fructose has the advantages that it is uncharged (thus eliminating the pH effects) and inexpensive and is a fermentable substrate at low oxygen tensions. The results for the 50-ml batch culture were striking: only a slight increase of pH was observed throughout the growth of the culture; the final cell density (6.7×10^8 cells per ml) was nearly twice that obtained with Sistrom medium A (Fig. 1a); and, most importantly, the final yields of chromatophores (A_{880}/A_{660} = 1.12) were almost comparable to those obtained from anaerobic cultures growing photoheterotrophically under low-intensity light conditions (A_{880}/A_{660} ratio = 1.14) (Fig. 1d) and twofold higher than those obtained under high-intensity light conditions (data not shown). Significantly, in the presence of fruc-

tose the addition of HEPES proved unnecessary for the control of pH: the final pH at 40 h was pH 7.27 (Fig. 1c).

The two-substrate system proved equally successful for the 20-liter batch culture. During the first 24 h following inoculation with a 10% inoculum, O_2 was rapidly consumed until it reached approximately 1% at 20 h and then maintained at a low level by control of the O_2 input valve. Growth then continued exponentially for a further 24 h, whereupon the cells were harvested (A_{660} [1 cm] = 4.0, A_{880}/A_{660} = 0.9) to yield approximately 180 g of wet cell paste. Only minor corrections to the pH had to be made during the 20-liter batch run.

The two-substrate modified Sistrom medium (henceforth called M2SF medium) allows *R. rubrum* to be grown routinely and reproducibly in large semiaerobic batch cultures with a minimum amount of control and expertise. By using M2SF medium, very high cell densities can be achieved and the yield of photosynthetic membranes approaches the maximum obtainable after photoheterotrophic growth under low-intensity light conditions. At present we envisage almost no upper limit to the size of the culture which can be grown in this way.

The fermentation of fructose by *R. rubrum* has been examined in great detail previously (6, 7). Schön (6) demonstrated a high level of BChl synthesis when *R. rubrum* was grown with 0.4% fructose as the sole carbon and energy source and showed that the adaptation to growth on fructose was preceded by a lag phase which could be eliminated if the organism was allowed to grow aerobically beforehand. Schultz and Weaver (7) demonstrated that 0.05% CO_2 was necessary to initiate the adaptation to fructose and that cell densities of 3.5 $\times$ 10^8 cells per ml could be obtained with this substrate.

The growth characteristics of *R. rubrum* with the succinate-fructose medium described here are consistent with the observations described above. The biphasic A_{880}/A_{660} profile shown in Fig. 1d, together with the rate of O_2 consumption in large-scale batch culture (data not shown), suggests that in the initial growth phase, succinate is preferentially consumed by aerobic chemoheterotrophic metabolism for up to 20 h, whereupon fructose is then metabolized, principally by fermentation. The storage products and CO_2 generated by aerobic metabolism probably abolish the lag phase noted by Schön (6) during adaptation to the fermentation of fructose. However, two observations suggest that with M2SF medium the metabolism of *R. rubrum* is probably bimodal, i.e., fermentative metabolism and semiaerobic respiration occur simultaneously. (i) The pH rise before and after 20 h is slow but continuous, even in the absence of added HEPES. This suggests that the uptake of protons during aerobic metabolism is being largely compensated for by acidification due to fermentation. (ii) In large-scale batch cultures, when the entry of O_2 is prevented for 20 h, growth slows considerably and a decrease in pH is observed.

The photosynthetic membrane of the phototrophic bacterium *R. rubrum* may offer novel opportunities for the overexpression of membrane or periplasmically located proteins. The medium described here, which maximizes photosynthetic membrane biogenesis but obviates the necessity for illumination, may open up the possibility for the development of this organism as a tool for biotechnology.

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