0273-2289/95/5152—0399$06.60

Optimization of a Chemically Defined, Minimal Medium for *Clostridium thermosaccharolyticum*

SUNITHA BASKARAN,
DAVID A. L. HOGSETT, AND LEE R. LYND*

Thayer School of Engineering,
Dartmouth College, Hanover, NH 03755

ABSTRACT

This article presents results from a systematic study aimed at formulating a defined, minimal medium for the growth of *Clostridium thermosaccharolyticum* in batch and in continuous culture. At least one vitamin appears to be essential, and there is no demonstrable requirement for trace minerals. The defined medium is shown to support growth on high substrate concentrations with scaled nutrient levels and is expected to permit complete utilization when nutrient limitation(s) are overcome. The observed elemental requirements are compared with cell mass fraction measurements and with a typical cell composition. The maximum growth rate (μ_{max}) for batch growth of *C. thermosaccharolyticum* on the minimal medium is 0.27 h^{-1} as compared with values of ~0.4 h^{-1} typically reported for growth on complex media. However, exponential growth terminates at an optical density of about 0.22 corresponding to about 40% of the final value attained. Greater understanding of nutrient requirements and interactions is needed to address this issue.

Index Entries: Ethanol; defined medium; minimal medium; optimization; *Clostridium thermosaccharolyticum.*

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Thermophilic bacteria have often been proposed for ethanol production from cellulosic biomass. Cellulase production and pentose fermentation are two important advantages of thermophiles relative to more conventional ethanol-producing organisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*. *Clostridium thermosaccharolyticum* is an obligately anaerobic, thermophilic bacterium with a gram-positive-type cell wall. It is frequently considered in the context of ethanol production from cellulosic biomass for its ability to consume pentoses rapidly, such as xylose and arabinose, particularly in coculture with a thermophilic, cellulolytic organism, such as *Clostridium thermocellum* (*1,2*).

Despite these advantages, the maximum concentration of ethanol reported for thermophiles is on the order of 25–30 g/L (*1, 3–6*), and ethanol production by thermophilic bacteria has yet to be demonstrated under realistic process conditions with production of readily recoverable concentrations of ethanol. Low ethanol tolerance has been cited as a possible explanation for the limited endogenous ethanol production; however, recent results from our laboratory indicate that the tolerance of *C. thermosaccharolyticum* to exogenously added ethanol is comparable to that of more conventional ethanol-producing organisms and is unlikely to significantly constrain its use for ethanol production (*7*). A second explanation is that endogenous ethanol production is curtailed by nutrient limitation(s) at high substrate concentrations. The above phenomena can be mistaken for each other; for example, inability to use high substrate concentrations could be attributed to ethanol inhibition when it was actually the result of nutrient limitation.

Various media, both complex and defined, have been used for cultivation of thermophiles. For *C. thermosaccharolyticum*, the only previous defined medium we are aware of is that of Hill et al. (*8*). For *C. thermocellum*, various defined media have been reported (*9–11*). This study focuses on formulation and optimization of a defined, minimal medium for growth of *C. thermosaccharolyticum* in batch and continuous culture. This is seen as a useful step toward formulation of a practical growth medium that will allow utilization of high substrate concentrations, thereby extending the concentration of ethanol produced by thermophiles.

MATERIALS AND METHODS

Organism and Fermentation Conditions

The source of *C. thermosaccharolyticum* HG8 was as described earlier (*4*). All chemicals used were of reagent grade (Sigma Chemical Co., St. Louis, MO).

Batch Culture

Batch experiments employed distilled water that had been further purified using a series of three ion-exchange columns. All experiments used a 2% (v/v) inoculum and were performed at 60°C in a reciprocal water bath shaker (New Brunswick Scientific Company, Edison, NJ). Ten grams per liter of MOPS (Morpholino propane sulfonic acid, sodium salt) were used as pH buffer. The pH at the start of batch experiments was 7.0 and did not fall below 6.6. The concentrations of xylose, rezasurin, MOPS, and l-cysteine hydrochloride were kept constant in all batch experiments, whereas the concentrations of macronutrients and/or vitamins were varied as described.

Continuous Culture

Distilled water was used in all cases. Continuous-culture experiments were performed at pH 6.0. MOPS buffer was eliminated from media for continuous culture, since buffering owing to KH_2PO_4 and CO_2-derived bicarbonate was found to be sufficient.

Quantification of Substrate, Cells, and Fermentation Products

Samples from the reactor were collected in tubes cooled in a beaker filled with ice so as to ensure that growth of the culture was arrested immediately on leaving the reactor. Culture optical density was determined in a Milton Roy model 21 spectrophotometer at 660 nm in tubes having a 1.8-cm path length. The extinction coefficient of 0.51 g cells/L/OD_{660} reported by Hill et al. (*8*) was used to calculate cell concentration. Quantification of xylose, ethanol, acetic acid, lactic acid, and propanediol was accomplished by HPLC, as previously described (*4*). Carbon recovery was calculated assuming 1 mol of CO_2 was produced/mol ethanol, acetic acid, and propanediol produced and neglecting cells, using the equation:

$$\text{Carbon recovery} = [(C_L*3) + (C_A*3) + (C_P*3) + (C_E*3)] / [(C_{X_0}*5) - (C_X*5)] \quad (1)$$

where C_L, C_A, C_P, and C_E represent concentrations of lactic acid, acetic acid, propanediol, and ethanol in the effluent stream, C_X and C_{X_0} represent concentration of xylose in the feed stream and effluent stream, respectively. Carbon recoveries of 85–90% (not accounting for cells) were typically observed.

Continuous Fermentation

Continuous fermentation of xylose was carried out in a 500-mL round-bottomed custom fermentation vessel (NDS, Vineland, NJ) with a 340-mL working volume. The reactor, pH probe, and all associated tubing and drip tubes were autoclaved for 1 h prior to use. The reactor configuration

was similar to that of Lynd et al. (4). pH was maintained at 6.0 by addition of 15 wt% potassium hydroxide to the fermentation vessel through a single-speed (100 rpm) peristaltic pump (Cole Palmer) controlled by an Applikon bioprocess controller. Autoclavable pH probes extended down through the rubber stopper and were obtained from Phoenix Electrodes, Houston, TX (model number G 05993-95) Masterflex programmable peristaltic pumps (Cole Palmer, model number 7500-90) were used to control the feed flow rate. Size 13 pump heads and tubing were used for feed delivery. Pumps were recalibrated each time flow rates were changed or new tubing was used. Feed carboys were connected to the fermentor with Luer Lok fittings. Drip tubes were used on sample lines to prevent contamination. Feed carboys were maintained under slight positive pressure with filter-sterilized nitrogen.

Fermentation was started in batch mode at approx 0.5% xylose concentration, with the fermentor maintained at 60°C and pH 7.0. Ten milliliters of a stock culture were transferred aseptically via syringe from a batch tube into the reactor through a septum in the reactor stopper.

RESULTS

Defined, Minimal Medium Development in Batch Culture

The mixture of nutrients in DM1 medium (Table 1) was found to support satisfactory growth of *C. thermosaccharolyticum* in batch culture. The quantities of macronutrients were calculated based on a typical cell composition for bacteria (*12*) and cell yield for *C. thermosaccharolyticum* (*8*) assuming complete utilization of 5 g/L xylose, and were then scaled by a factor of 5 so as to be present in excess of the stoichiometric requirement relative to the substrate. Various nitrogen sources were tested; ammonium was the preferred nitrogen source relative to urea (data not shown) as evidenced by a shorter lag phase and faster growth on ammonium-based media.

The concentrations of all macronutrients and vitamins were varied simultaneously as the first step toward determining the stoichiometric requirement for individual nutrients, that is, the amount of nutrient required per unit cell synthesized. The optical density at 660 nm was monitored at frequent intervals as a measure of growth. Maximum optical density (OD) was typically attained 2–3 d after inoculation. The calculated requirement of all nutrients at the nominal concentration (arbitrarily assigned a value of 1) was sufficient for complete xylose utilization, as suggested by no significant increase in culture turbidity at higher nutrient levels and the undetectable level of xylose in the culture after growth had ceased (data not shown). The maximum OD at the 0.2 level of nutrients was lower, which suggests that at least one of the nutrients becomes limiting at that level.

Table 1
Composition of Defined Media for *C. thermosaccharolyticum**

Nutrient	Concentration in DM1 medium, g/L	Concentration in DM2 medium, g/L	Concentration in DM3 medium, g/L
Carbon and energy source (g/L)			
d-Xylose	5.0	10	10
Buffers (g/L)			
MOPS, sodium salt	10.0	0	0
L-cysteine $HCl \cdot H_2O$	1.0	1.0	1.0
Macronutrients (g/L)			
KH_2PO_4	0.06	3	0.6
Na_2SO_4	0.02	1	0
$MgCl_2 \cdot 6H_2O$	0.02	1	0.2
$CaCl_2 \cdot 2H_2O$	0.01	0.5	0.1
$FeCl_2 \cdot 4H_2O$	0.002	0.01	0.02
NH_4Cl	0.2	10	0
$(NH_4)_2SO_4$	0	0	0.4
Vitamins (mg/L)			
Pyridoxamine Dihydrochloride	2.0	20	1
p-Amino benzoic acid	0.4	4	0.2
d-Biotin	0.2	2	0.1
Vitamin B_{12}	0.2	2	0.1
Thiamine	0.4	4	0.2

*In all cases, the medium contained 0.5 mL of 0.2 wt% Rezasurin/Liter water. DM2 and DM3 medium also contained 0.01 mL of Mazu/L as defoamer.

We then varied the concentrations of nutrients, taken one at a time (the vitamins were treated collectively as one nutrient) so as to determine stoichiometric requirements of individual nutrients. For this experiment, the inocula consisted of cultures that were starved for a particular nutrient by means of two successive transfers into medium containing 0.2 levels of all nutrients followed by a single transfer into medium that contained a 0.2 level of the particular nutrient and 1 levels of all other nutrients. Results from the experiment are presented in Fig. 1.

For magnesium chloride, ferrous chloride, potassium phosphate, and ammonium chloride, the 1 level was found to be sufficient, whereas lower resulted in reduced growth. For sodium sulfate, calcium chloride, and the vitamins, there was no significant decrease in extent of growth at any of the levels tested, including in medium that completely lacked these nutrients. For sodium sulfate and calcium chloride, three further transfers into

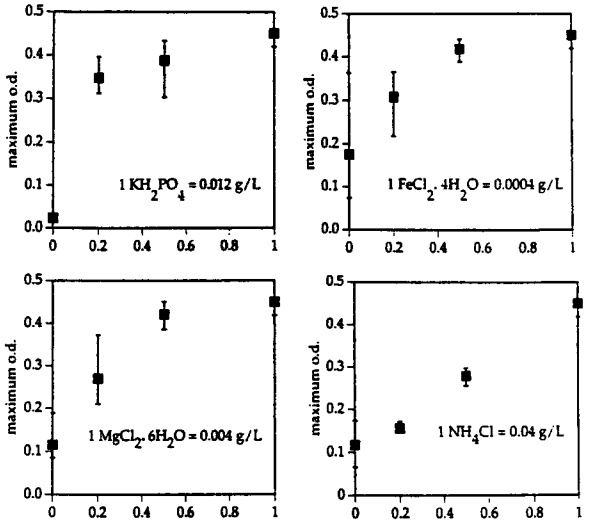

Fig. 1. Determination of minimal macronutrient requirements in batch culture. Horizontal axis represents fraction of estimated stoichiometric requirement of nutrient. Values represent mean and error bars represent range based on four data points at each condition.

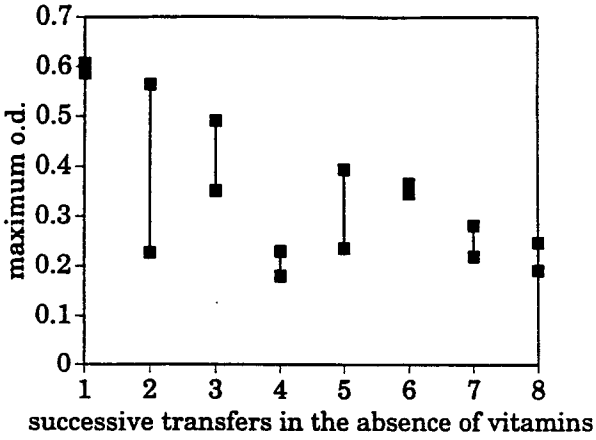

Fig. 2. Batch growth of *C. thermosaccharolyticum* in the absence of vitamins. Error bars represent range based on duplicates or triplicates.

medium that completely lacked these nutrients were performed without detecting any reduction in maximum extent of growth attained.

Eight successive transfers were performed for the vitamins (treated as a group). As shown in Fig. 2, the maximum optical density attained decreased by 50% after eight transfers. Neglecting intracellular accumulation, the only possible source of vitamins in media was carryover from inoculum. After eight transfers, carryover was $< (0.02)^8$ of the inoculum

Table 2
Steady-State Data for *C. thermosaccharolyticum* on Defined Media with Complete Utilization of 10 g/L Xylose

Medium	Dilution rate, h^{-1}	OD_{660}	Ethanol, g/L	Acetic acid, g/L
DM2	0.0477	1.43	2.97	1.57
DM2	0.0653	1.12	3.23	1.63
DM2—Na_2SO_4 + $MgSO_4$	0.0477	1.18	3.20	1.60
DM2—Na_2SO_4 – NH_4Cl + $(NH_4)_2SO_4$	0.0477	1.14	3.09	1.14
DM3	0.0459	0.73	3.50	1.78
DM3	0.0653	0.92	3.10	1.73

for the first transfer. The persistence of significant growth is rather puzzling in light of the large dilution on subsequent transfers. Nevertheless, the trend of declining optical density does support a requirement for at least one vitamin. Maximum optical density for vitamin-starved cultures was attained within 2–3 d after inoculation, which is similar to that for nonstarved cultures.

Defined, Minimal Medium Formulation for Continuous Culture

DM2 medium with 10 g/L xylose as limiting substrate was used as a starting point for medium optimization in continuous culture. The medium was formulated to contain significant excess of all nutrients after accounting for the higher substrate concentration in the medium and the correspondingly higher cell concentration expected. MOPS buffer was eliminated, since bicarbonate buffering from CO_2 produced was expected to provide sufficient buffering. Complete utilization of xylose was observed at dilution rates of 0.0477 h^{-1} and 0.0653 h^{-1} (Table 2). Having established that the concentrations of minerals and vitamins in DM2 medium were sufficient for complete xylose utilization, we attempted to study the requirement for individual nutrients in continuous culture.

Vitamin Requirement

The feed stream to a reactor that was at steady state at a dilution rate of 0.0653 h^{-1} on DM2 medium was changed in step fashion to DM2 medium lacking vitamins at time = 58 h as shown in Fig. 3. The effluent xylose concentration remained at undetectable levels until about 42 h after the step change had been initiated, after which the xylose concentration increased rapidly and wash-out of the culture occurred.

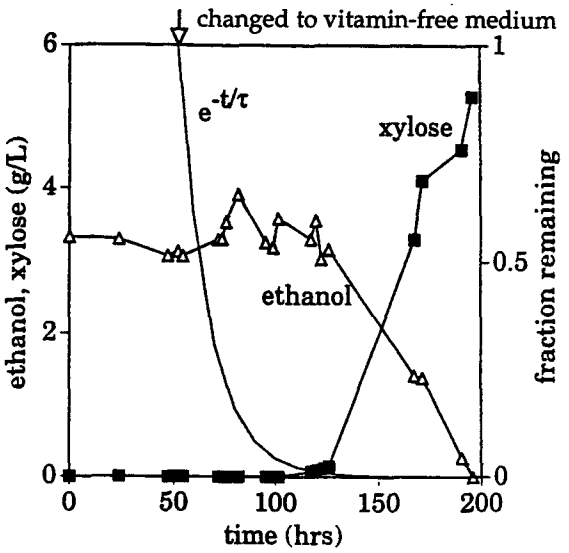

Fig. 3. Continuous cultivation of *C. thermosaccharolyticum* in the absence of vitamins τ = 15.3 h. —△ Changed to vitamin-free medium.

This confirms batch results, indicating that vitamins are required for growth of *C. thermosaccharolyticum* on DM2 medium. Furthermore, the inability to detect effluent xylose until 42 h after the change to vitamin-free medium was made suggests that the requirement for vitamins is likely to be much less than the quantity supplied. Assuming first-order decay of vitamin concentrations in the fermentor owing to dilution, the fraction of the original vitamins remaining after 42 h is 0.0642 (as shown below).

Fraction remaining after time t:

$$(C / C_0) = \exp - (t/\tau) \quad (2)$$

where C = concentration of nutrient at time t, C_0 = concentration of nutrient at time 0, and τ = residence time (= 15.3 h).

This is a conservative estimate of the actual vitamin requirement, since depletion owing to consumption by the organism has not been accounted for. Thus, it appears that ~6% of the original vitamin concentrations may be sufficient for growth at a dilution rate of 0.0653 h^{-1}. This requirement is significantly higher than that for batch culture. The kinetic requirement for vitamins in continuous culture may be a partial explanation for the discrepancy.

Elimination of Sodium

As mentioned earlier, we were unable to demonstrate a requirement for sodium in batch culture after successive transfers in the absence of sodium. In batch experiments, sodium sulfate was the sole source of sodium (with the exception of MOPS). In order to study the requirement

of sodium in continuous culture, sodium sulfate was eliminated from DM2 medium, and magnesium sulfate (1.74 g/L) was added to the medium (in addition to the magnesium chloride in DM2 medium). All other nutrients were as per DM2 medium. The modified sodium-free medium was supplied as feed to a fermenter that was previously at steady state at a dilution rate of 0.0477 h^{-1} on DM2 medium.

After steady-state conditions were attained, the feed was again changed to DM2 medium without sodium sulfate; this time, however, ammonium chloride was replaced with ammonium sulfate (2.74 g/L).

Steady-state data for the above-described conditions (*see* Table 2) suggest that sodium is not a required component in the DM2 medium. This observation is in agreement with the literature in that a sodium requirement is generally difficult to establish owing to its presence as a contaminant (*13*).

Minimal Medium Optimization

DM3 medium (Table 1) is a modification of DM2 medium without sodium sulfate. Sulfate is instead supplied as ammonium sulfate, and the concentrations of vitamins have been reduced to incorporate information obtained from the experiments described above. The concentrations of all other nutrients are derived from the minimal medium established in batch culture after correcting for the higher cell concentration in continuous culture. As shown in Table 2, complete xylose utilization was obtained, which suggests that fermentation performance on DM3 medium is comparable with that on DM2 medium (although growth on DM3 medium is slightly reduced), while containing lower nutrient concentrations in many cases.

Continuous Cultivation at High Substrate Concentrations

Medium for growth on 50 g/L xylose was based on DM3 medium, with the concentrations of macronutrients and vitamins scaled by a factor of 5 to account for the higher substrate concentration. Continuous feeding was initiated at time zero at a dilution rate of 0.0282 h^{-1} as shown in Fig. 4. Ethanol, acetic acid, and lactic acid were produced during the fermentation. Ethanol production increased steadily reaching a peak of 10.53 g/L at 95.33 h, but decreased thereafter to 1 g/L at 198.33 h. In order to test for nutrient limitation, a pulse of all macronutrients and vitamins was added to the fermenter so as to increase the concentration in the fermenter by twice that in the feed. Once again, product concentrations increased rapidly with production of 11.33 g/L ethanol at 263 h before falling, thereby indicating that the culture was nutrient-limited.

Having established nutrient limitation, the concentration of ammonium sulfate in the feed was doubled at 339.5 h to determine if the limiting nutrient was nitrogen. Product and cell concentrations increased in response to the step change, indicating nitrogen limitation. Such a pulse

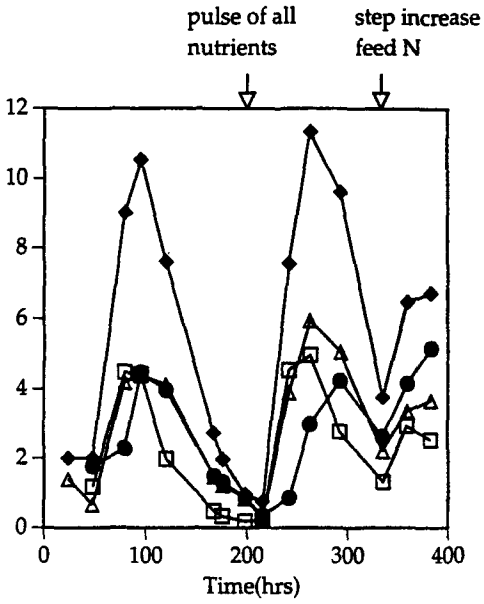

Fig. 4. Continuous cultivation of *C. thermosaccharolyticum* at high substrate concentrations. —□— OD (660 nm), —●— lactate (g/L), —△— acetate (g/L), and —▲— ethanol (g/L).

and shift approach is expected to be useful for eliminating nutrient limitation and formulating a medium that will permit utilization of high substrate concentrations.

DISCUSSION

Analysis of Elemental Requirements

Table 3 presents observed requirements of various elements calculated from the batch minimal medium presented earlier using the cell yield of Hill et al. (*8*). The observed requirements are compared with:

1. Measured elemental mass fraction of N and P reported by the same authors for *C. thermosaccharolyticum;*
2. Observed requirements for continuous cultivation of *C. thermocellum* ona defined medium; and
3. A typical cell composition, all presented in units of g nutrient/g cell.

The requirement for N compares well with the measured elemental mass fraction and the typical cell composition. The higher requirement for *C. thermocellum* may reflect volatilization losses in continuous culture. For P

Table 3
Analysis of Elemental Requirements for *C. thermosaccharolyticum*

Element	Observed requirement in batch, g nutrient/g cell	Measured elemental mass fraction, g nutrient/g cell*	Observed requirement for *C. thermocellum*	Typical cell composition, g nutrient/g cell
N	0.1046	0.121	0.1981	0.14
P	0.0273	0.0094	0.034	0.03
K	0.0345	—	0.043	0.01
Ca	none	—	0.0027	0.005
Mg	0.0048	—	0.0081	0.005
Fe	0.0011	—	<0.0008	0.002

—Did not measure.
*Measured elemental mass fraction for *C. thermosaccharolyticum* on defined medium at 0.067 h^{-1} (*7*). Observed requirement for *C. thermocellum* at 0.167 h^{-1} on 25 g/L fructose-based defined medium with all other nutrients as per Klapatch et al. (*11*) (Hogsett, D. A. L.; unpublished data). Typical cell composition modified from Bailey and Ollis (*12*).

and Mg, the observed requirements are in good agreement with the typical cell composition. The observed requirement for K is comparable for both organisms, while being higher than in the typical cell.

Such analyses can be used to differentiate stoichiometric and kinetic requirements. Kinetic requirements become important in continuous culture, where the concentration of a nutrient has to be much higher than the value of the corresponding saturation constant (k_s) in order for growth to proceed at the maximum rate. It is interesting to note that there is no demonstrable requirement for trace minerals in batch or in continuous culture.

Growth Rate on Defined Media

The maximum growth rate μ_{max} measured for *C. thermosaccharolyticum* growing in batch mode on DM1 medium containing 1 level of nutrients is 0.27 h^{-1} as compared with values of ~0.4 h^{-1} typically reported for growth on complex media. However, exponential growth on DM1 medium terminates at an OD of about 0.22 corresponding to about 40% of the final value attained on 5 g/L xlyose. The hypothesis that exponential growth terminates owing to the onset of kinetic limitation for a nutrient was tested, but could not be supported since growth on media containing 2 and 5 levels of nutrients was similar to that on the minimal medium. We then examined the possibility that the premature termination of exponential growth was the result of kinetic limitation for a trace mineral that was present as a contaminant in the medium. A mixture of trace minerals (*8*)

was added to DM1 medium; once again there was no signficant difference in the pattern of growth. We are currently studying the medium formulation of Hill et al. (*8*), who reported steady-state growth of *C. thermosaccharolyticum* at dilution rates of up to 0.33 h^{-1} on a defined medium to identify the limitation.

CONCLUSIONS

A chemically defined, minimal medium has been formulated for growth of *C. thermosaccharolyticum* in batch and continuous culture. At least one vitamin appears to be essential, and there is no demonstrable requirement for trace minerals. The defined medium is shown to support growth on high substrate concentrations with scaled nutrient levels and is expected to permit complete utilization when nutrient limitation(s) are overcome. The observed elemental requirements are in good general agreement with cell mass-fraction measurements and with a typical cell composition. The observed requirements for *C. thermosaccharolyticum* are also compared with those for continuous cultivation of *C. thermocellum* on a defined medium. Exponential growth on the defined medium terminates at an OD of about 0.22 corresponding to about 40% of the final value attained and needs to be addressed through further study of nutrient requirements. Such information on defined, minimal medium formulation is needed for developing practical media that support growth at high substrate concentrations with production of readily recoverable concentrations of ethanol.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of the National Renewable Energy Laboratory (subcontract XAP 3-11195-01). Expert technical assistance was provided by Patrick Riehl and Keith Levenson.

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