0273-2289/96/5758—0307$10.00

Studies on Nutrient Requirements and Cost-Effective Supplements for Ethanol Production by Recombinant *E. coli*

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ABSTRACT

This article describes a systematic study of the nutritional requirements of a patented recombinant ethanologenic *Escherichia coli* (11303:pLOI297) and provides cost-effective formulations that are compatible with the production of fuel ethanol in fermentations of lignocellulosic prehydrolysate characterized by high xylose conversion efficiency. A complex and nutrient-rich laboratory medium, Luria broth (LB), provided the benchmark with respect to fermentation performance standard. Xylose fermentation performance was assessed in terms of the target values for operational process parameters established by the US National Renewable Energy Laboratory (NREL)—final ethanol concentration (25 g/L), xylose-to-ethanol conversion efficiency (90%), and volumetric productivity (0.52 g/L·h). Biomass prehydrolysates that are rich in xylose also contain acetic acid, and in anticipation of a need to reduce acetic acid toxicity, the fermentors were operated with a pH control set-point of 7.0 Growth and fermentation in the minimal defined salts (DS) medium was only about 15% compared to the reference medium. Amendment of the minimal medium containing 6 wt% xylose with both vitamins and amino acids resulted in improved growth, but the volume productivity (0.59 g/L·h) was still only about 54% of that with LB (1.1 g/L·h). Formulations directed at cost reduction through the use of less expensive commercial complex nutritional supplements were within 90% of the NREL process target with respect to yield and provided a productivity at about 80% of the LB medium, but were not economical. Corn steep liquor (CSL) at about 7–8 g/L was shown to be a complete source of nutritional requirements and supported a fermentation performance approaching that of LB. At a cost of CSL of $50/t (dry wt), the economic impact of using this amount CSL as the sole nutritional supplement in a cellulosic ethanol plant was estimated to be about 4¢/gal of ethanol.

Index Entries: Xylose; recombinant *E. coli*; fuel ethanol; corn steep liquor; nutrient requirements.

Abbreviations: LB, Luria broth; sLB, LB supplemented with 0.5 m*M* Mg + 17 m*M* PO_4; mLB, 2.5 g/L YE + 2.5 g/L Tryptone; DS, Defined salts medium; YE, Difco yeast extract (g/L); VYE, Veeprex B430 yeast extract (g/L); Tryp, Bacto-

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tryptone (g/L); Pan, pancase S (g/L); TW, Toronto tap water; AP, 1.4 g/L ammonium monohydrogenphosphate; CSL, corn steep liquor (Nacan Products) (mL/L); Vit, Vitamin stock (added 5 mL/L) for 2X Vit—added 10 mL/L; Glut, 0.25 g/L glutamic acid (g/L); AA, amino acid cocktail = 50 mg/L each of aspartic acid, tyrosine, tryptophan, phenylalanine, and histidine; AA^+, 50 mg/L each of arginine, asparagine, aspartic acid, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

INTRODUCTION

The economics of the large-scale production of fuel ethanol are very sensitive to the cost of fermentation feedstock, and consequently, less expensive lignocellulosic biomass and waste materials are being targeted as alternatives to cereal or sugar crops *(1,2)*. However, lignocellulosic materials remain recalcitrant to bioconversion because the yeast cultures presently employed in the starch-based fermentation ethanol industry are unable to utilize the five-carbon pentose sugars derived from the hydrolysis of hemicellulose *(3)*. Attention has been focused on D-xylose, because this pentose sugar represents a major constituent of the hemicellulose in the particular biomass species (energy crops) and waste resources presently being considered as potential fermentation feedstocks *(3,4)*.

Current processing technologies employing dilute sulfuric acid are capable of yielding a "prehydrolysis" stream containing a maximum xylose concentration (without concentrating) of about 60 g/L *(5)*. Current research efforts are directed to bioprocesses capable of producing fuel ethanol from hemicellulose hydrolysate at a pentose conversion efficiency of >70%, thereby surpassing that of current bioconversion technologies that propose to use pentose-fermenting yeasts *(6,7)* or thermophilic bacteria, which generally suffer from relatively low tolerance to ethanol *(8)*.

Although the bacterium *Escherichia coli* is capable of utilizing all biomass-derived hexose and pentose sugars, the product of anaerobic metabolism is a mixture of organic acids with ethanol also sometimes appearing as a fermentation end product *(9–14)*. A recombinant that expresses *Zymomonas mobilis* genes for pyruvate decarboxylase and alcohol dehydrogenase II (termed the *pet* operon) *(15)* on a multicopy plasmid designated pLOI297 *(16,17)* has been shown to convert both hexose and pentose sugars to ethanol at near-maximum theoretical efficiency *(14,18–23)*. The physiological characteristics of this plasmid-bearing recombinant (*E. coli* B ATCC 11303:pLOI297) has been extensively investigated *(17–23)*. However, the majority of this research has been conducted using a complex and nutrient-rich medium (LB) composed of laboratory-grade chemicals *(17–20)*. The use of expensive nutrient supplements has a serious negative impact on the economics of ethanol production. The extent to which these complex nutrients are essential for high-performance fermentation is not known.

The objective of this study was twofold. First, to establish the basic nutritional requirements of recombinant *E. coli* (11303:pLOI297) using defined media with LB as the reference for comparative fermentation performance in terms of both ethanol yield and productivity in pH-controlled batch fermentations with xylose as substrate, and, second, by employing inexpensive complex nutritional supplements, to formulate a cost-effective medium compatible with high-performance fermentation in a full-scale commercial fuel ethanol production plant.

Criteria for Assessing Fermentation Performance of the Biocatalyst

Cost reduction is the primary driving force for process improvement and alternative feedstock utilization. Techno-economic sensitivity analyses related to fuel ethanol production from cellulosic biomass have revealed the ordered importance of certain bioconversion process parameters whereby the price of ethanol was most sensitive to yield *(5)*. The efficient utilization of the hemicellulose component of lignocellulosic feedstocks offers an opportunity to reduce significantly the price of ethanol. In terms of the fermentation portion of the overall production process, the ethanol yield is a function of the efficiency with which the biocatalyst converts sugar to ethanol. The price of ethanol was also found to be sensitive to the final concentration of ethanol in the bioreactor, but was less sensitive to the rate of ethanol production *(5)*. Such economic analyses establish the criteria for assessing the performance of the process biocatalyst as ethanol yield, ethanol concentration and productivity. In the case of xylose utilization, the National Renewable Energy Laboratory (NREL) (Golden, CO) has established target values for each of these fermentation process parameters *(4)*. The target for ethanol yield (based solely on xylose) is 0.46 g/g, which is equivalent to a xylose-to-ethanol conversion efficiency of 90% (relative to the theoretical maximum conversion efficiency). The objective in terms of ethanol concentration is 25 g/L and for volumetric productivity it is 0.52 g ethanol/L·h *(4)*.

Elemental Composition of Cell Mass as a Nutritional Indicator

Microorganisms exhibit growth optima with respect to both physical and chemical environmental factors. A prerequisite to growth is that the aqueous environment ("medium") supply the elements of carbon, nitrogen, phosphorous, and sulfur that, in addition to hydrogen and oxygen, are the major components of all biomolecules *(24)*. In addition to sodium, potassium, magnesium, calcium, and iron, certain minerals are required in relatively much smaller amounts and are therefore referred to as "trace elements" *(24)*. These trace elements are primarily the metals such as manganese, copper, cobalt, molybdenum, zinc, and so forth, that act as enzyme cofactors. In addition, there are can be certain other essential elements that, apart from the known "vitamins," are referred to collectively as "growth factors" *(24)*. In general, the elemental composition of the organism provides insights into nutritional requirements. However, it is known that cellular composition can be influenced by the chemical nature of the growth environment. For example, the amount of NaCl in the medium has been shown to influence the sodium level in the cell mass *(25)*. Table 1 provides examples of the elemental composition both with specific reference to *E. coli* and bacteria in general.

In this study, we have focused attention principally on three major nutritional elements, namely nitrogen (N), phosphorus (P), and magnesium (Mg). Based on several literature sources relating to the elemental composition of *E. coli* cell mass *(24–27)*, we have assumed that, on average, the elemental composition with respect to N, P, Mg was constant at about 15, 1.5, and 0.15 wt%, respectively *(24–27)*. Furthermore, it has been assumed that the cellular composition reflects the mass ratio requirements of these various nutritional elements in the culture medium *(25)*. Consequently, it can be inferred that the minimal concentrations (g/L) of N, P, and Mg required to support a yield of 1 g dry wt cells/L, are 0.15, 0.015, and 0.0015, respectively (*see* Table 2).

Table 1
Elemental Composition of Bacteria (%dry wt basis)

Element	*E. coli*,[a]	Bacteria,[b] gram-negative	Bacteria,[c] in general
Carbon (C)	50		ca. 50
Nitrogen (N)	15	12	10
Phosphorus (P)	3.2	1.5	2.0–3.0
Sulfur (S)	1.1	0.33	0.2–1.0
Sodium (Na)	1.3		0.5–1.0
Potassium (K)	1.5	0.75	1.0–4.5
Magnesium (Mg)	0.5	0.15	0.1–0.5
Calcium (Ca)	1.0	0.10	0.01–1.1
Iron (Fe)	0.24	0.015	0.02–0.20
Manganese (Mn)	Trace	0.005	0.001–0.01
Copper (Cu)	Trace	0.001	0.01–0.02
Others	Trace	Trace	Trace

[a]Demain and Solomon *(25)*.
[b]Pirt *(24)*.
[c]Aiba et al. *(11)*.

Selection of Conditions for Assessing Fermentation Performance

E. coli is less ethanol tolerant than yeast or *Zymomonas (28)*, and for this reason, a concentration of 40 g/L D-xylose was selected for routine fermentation performance assessment with different media formulations because the anticipated maximum ethanol concentration would fall below the inhibitory threshold for *E. coli*, thereby avoiding unnecessary complications in interpretation of growth and fermentation kinetics.

The *pet* operon (plasmid pLOI297) contains antibiotic resistance markers for both ampicillin and tetracycline *(17)*. Since a recognized contribution to ethanol yield reduction in fermentations employing plasmid-bearing recombinant *E. coli* relates to the potential for genetic instability *(29,30)*, all media formulations used in this investigation included antibiotics.

Acetic acid toxicity is an obstacle to the fermentation of biomass hydrolysates *(31)*, but since the inhibitory effect on recombinant *E. coli* is minimized at elevated pH *(32)*, a pH value of 7.0 was selected as the control set-point.

The "Reference" Medium

The nutritionally-rich, complex LB medium has been commonly used in studies with recombinant *E. coli (17–20)*. However, LB medium is known not to support carbon-limited growth at sugar concentrations greater than about 30 g/L *(21,33)*. Carbon-limited growth at 40 g/L can be achieved with LB supplemented with mg and phosphate, and for this reason, this amended LB (designated as "sLB") was selected as the reference medium in this study and provided a performance "benchmark" for purposes of comparing fermentation performance of the recombinant in variously formulated media. We have previously described the formulation of a defined minimal medium that is compatible with growth of recombinant *E. coli* using pure xylose as the sole carbon source *(33)* and this medium

Table 2
Composition of Different Fermentation Media
with Respect to the Potentially Limiting Elements–N, P, mg

Designation of medium, formulation/composition	Elements, g/L		
	N	P	Mg
Min. amount required (g/L) per g (dry wt.) biomass[a]	0.15[b]	0.015[c]	0.0015[c]
LB	1.76	0.09	0.006
sLB	1.76	0.62	0.018
mLB	0.56	0.03	0.002
DS	0.59	0.62	0.020
DS + Vit + 0.25 g/L Glut	0.61	0.62	0.020
DS + Vit + 0.25 g/L Glut + AA*	0.76	0.62	0.020
TW + AP + 5.0 g/L VYE	0.72	0.35	0.012
TW + 37 mL CSL (15.7 g)	1.04		0.105

The N content of media is based on the determination of "total N."
[a]Based on elemental composition of *E. coli* dry biomass.
[b]Demain and Solomon *(25)* p.108.
[c]Pirt *(24)* pp. 123,124.

(designated as DS) provides a base case for modifications designed not only to examine basic nutritional requirements, but also to improve growth rate and ethanol productivity.

MATERIALS AND METHODS

Organism

The patented recombinant *E. coli* B (ATCC 11303 carrying the *pet* plasmid pLOI297) *(16,17)* was a gift from L. Ingram (University of Florida, Gainesville, FL).

Long-Term Storage/Maintenance of Organism

Plasmid-bearing cultures, grown from single-colony isolates on selective agar medium, were stored at –10°C in LB medium supplemented with antifreeze (glycerol at 20 mL/dL) and sodium citrate (1.5 g/dL). The phenotypic characteristics of the recombinant culture were related to antibiotic (ampicillin and tetracycline) resistance, colony size, and morphology. The recombinant culture was recognized on selective media, containing the antibiotics ampicillin (40 mg/L) and tetracycline (10 mg/L), by the formation of distinctive large, yellowish, opaque colonies *(21,34)*.

Fermentation Equipment

pH-stat STR batch fermentations were conducted in a volume of 1500 mL in MultiGen (model F2000) (New Brunswick Scientific Co., Edison, NJ) stirred-tank bioreactors fitted with agitation, pH, and temperature control (30°C). The central agitator shaft was fitted with three multiblade turbine impellers, and the rotation speed was constant at about 100 rpm. The pH was monitored using a sterilizable combination pH electrode (Ingold) and was controlled at 7.0 by the addition of 4*N*

KOH. On average, about 75 mL of 4*N* KOH were added during the fermentation of 4 wt% xylose in STR pH-stat batch fermentations.

Methods of Preculture and Inoculation Procedures

A 1-mL aliquot of a glycerol/citrate preserved culture was removed from cold storage (freezer) and transferred to about 100 mL of complex medium (LB), containing about 2% xylose and supplemented with antibiotics (40 mg/L ampicillin and 10 mg/L tetracycline), in 125-mL screw-cap flasks and grown overnight at 30°C and low agitation in a water bath shaker. In the case of experiments employing defined media, the inoculum was prepared by transferring an aliquot of a glycerol/citrate preserved culture to DS medium containing about 2 wt% xylose and antibiotics (40 mg/L ampicillin and 10 mg/L tetracycline).

STR batch fermentations were inoculated by transferring approx 100 mL of the overnight flask culture directly to 1400 mL of medium in the bioreactor. The initial cell density was in the OD_{550} range of 0.1–0.2, corresponding to 30–50 mg dry wt cells/L.

For fermentations involving defined (minimal) media, an alternative preculture and inoculation procedure was developed ("plate direct inoculation") during the course of this investigation, which permitted preculture using LB, but avoided the typical centrifugal harvesting/washing procedure, which was found to be detrimental to culture vitality. This procedure also minimized the potential for transfer of nutrients during inoculation. Preculture was accomplished using LB (Ap/Tc) agar plates. A glycerol/citrate culture was used to inoculate several plates using a standard "spreading" technique. Following overnight incubation at 30°C, the culture lawns were scraped off the plates using a sterile inoculation loop, and the plates were rinsed with DS medium. The cell suspensions from several plates were pooled, agitated to achieve homogeneity, and used to inoculate the STR.

Fermentation Media

For comparative purposes, the nutrient-rich, complex culture medium described by Luria and Delbruck *(34)* was used as the benchmark standard. This medium is commonly referred to as "Luria broth" (LB) and consists of 5 g Difco yeast extract (YE), 10 g Difco Tryptone, and 5 g NaCl/L of distilled water *(34)*. Two different modifications of this LB medium were used in this investigation. sLB was fortified with magnesium (0.5 m*M* added as $MgSO_4$) and phosphate (17 m*M*). Modified LB (mLB) contained reduced amounts of YE (2.5 g/L) and Tryptone (2.5 g/L) and no NaCl.

In the case of fermentations with defined media, the DS medium was comprised of 2.25 g NH_4Cl, 0.1g $MgSO_4$, 0.7 mg $FeCl_3 \cdot 6H_2O$, 2.72 g KH_2PO_4, 3.48 g K_2HPO_4, 5.0 g NaCl, 12 mg $CaCl_2 \cdot 2H_2O$, 9.9 mg $MnCl_2 \cdot 4H_2O$, 0.05 mg thiamine, and 0.21 g citric acid/L of distilled water. In certain instances, the DS was fortified with glutamic acid (Glut) at a final concentration of 0.25 g/L. Two different amino acid supplements were used—one contained a mixture of six amino acids (aspartate, glutamate, histidine, phenylalanine, tryptophan, tryrosine—designated as AA) and the other contained a mixture of 17 amino acids (aspartate, arginine, asparagine, cysteine, histidine, glutamate, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine—designated as AA^+). The final concentration of each amino acid in the medium was 50 mg/L,

except glutamic acid, which was 250 mg/L. In the case of fermentations with semidefined media, the DS medium was supplemented with various complex nutrient supplements, including yeast extracts (YE Bacto-YE from Difco Laboratories, Detroit, MI; VYE Veeprex B430 YE from Champlain Industries Ltd., Mississauga, Canada) and casein hydrolysates (Tryp Bacto-Tryptone from Difco Laboratories, Detroit, MI; Pan–Pancase S from Champlain Industries Ltd., Mississauga, Canada).

Some formulations involved the use of tap water (TW) instead of distilled water and were supplemented with 1.4 g/L ammonium monohydrogenphosphate (AP) or various specified amounts of corn steep liquor (CSL) (Nacan Products Ltd., Collingwood, Canada).

Unless stated otherwise, the xylose concentration in all media was about 40 g/L. All media contained antibiotics (40 mg/L ampicillin and 10 mg/L tetracycline). All media were sterilized by autoclaving at 121°C for 30 min. Antibiotics were filter (0.45-μ pore size) sterilized and added to the sterilized medium after cooling.

Analytical Procedures

Growth was measured turbidometrically at 550 nm (1-cm lightpath) and culture dry weight was measured directly by microfiltration, washing and drying the filter to constant weight under an infrared heat lamp. Culture cell mass was estimated using the relationship 1.0 OD_{550} = 0.34 g dry wt cells/L *(14,20,35)*. Carbon balances (% carbon recovery) were calculated by the method previously described *(33)*. Compositional analyses of fermentation media and cell-free spent media were determined by HPLC using an HPX-H column (Bio-Rad Labs, Hercules, CA) as described previously *(33)*. The total nitrogen content of the complex nutrient supplements Bacto-YE, TRP, VYE, Pan, and CSL was calculated based on product specifications provided by the manufacturers. Inorganic elemental analysis of TW and the complex nutrient supplements was performed at the SLOWPOKE Reactor Facility (University of Toronto).

Determination of Growth and Fermentation Parameters

The mass-based growth yield coefficient ($Y_{x/s}$) was calculated by dividing the maximum cell density (dry wt cells/L) by the concentration of xylose consumed. The ethanol (product) yield ($Y_{p/s}$) was calculated as the mass of ethanol produced (final concentration)/mass of xylose added to the medium and was corrected for the dilution caused by the addition of alkali ($corY_{p/s}$). However, $Y_{p/s}$ was not corrected for the contribution from fermentable components other than xylose, which accounts for ethanol yield values that are greater than the theoretical maximum of 0.51 g EtOH/g xylose.

The average volumetric rate of xylose utilization (Q_s) was determined by dividing the initial xylose concentration by the total time required to achieve complete depletion of xylose from the medium. The maximum volumetric rate of sugar utilization ($max\ Q_s$) was estimated from the maximum slope in plots of sugar concentration vs elapsed fermentation time. The corresponding value of volumetric productivity (Q_p) was calculated by multiplying $maxQ_s$ by $corY_{p/s}$. Since the dry wt cell mass was only determined as an end point (i.e., at the time when the culture mass was maximal) and was not determined over the entire time course of the batch fermentation (other than indirectly as OD), the specific rates of xylose utilization and ethanol production were not routinely determined. However, in

cases where growth ceased considerably in advance of the complete utilization of the sugar, the linear rate of xylose utilization during the postgrowth phase ($maxQ_s$) could be divided by the maximum cell mass to yield an estimate of the specific rate of xylose utilization (q_s). Similarly, the specific productivity (q_p) was estimated by multiplying q_s by the overall ethanol yield ($corY_{p/s}$).

RESULTS AND DISCUSSION

Basic Nutritional Requirements

The first approach to gaining further insights into the basic nutritional requirements of recombinant *E. coli* was one of "subtraction refinements' of the complex reference medium (sLB). At a xylose concentration of 40 g/L, the maximum cell density under carbon-limiting conditions is 2 g dry wt cells/L *(33)*, and to support this cell density, the medium should contain minimally 0.3 g assimilable N, 0.03 g P, and 0.003 g Mg (Table 2). It should be noted that the value for the N content of the different media shown in Table 2 is based on a determination for "total N" and not "assimilable N." LB contains 5.0 g/L YE and 10 g/L Tryp and one particular reformulation of LB, designated as mLB, that contains 2.5 g/L YE, and 2.5 g/L Tryp has been recommended by others as a useful refinement for fermentations with recombinant ethanologenic *E. coli* cultures *(36,37)*. However, the utility of this formulation is dependent on the concentration of fermentable sugar. Whereas mLB with 40 g/L xylose contains sufficient N to support a cell density of 2 g/L, there is a potential for insufficiency with respect to both P and Mg (Table 2). Figure 1 compares the effect of various subtraction refinements of sLB on the calculated total N content (A), the xylose-based growth yield (B), the ethanol yield (C), and the volumetric productivity (D). The arrow in Fig. 1A indicates the minimum requirement for assimilable N (0.3 g/L), and the arrows in Figs. 1C,D indicate the NREL targets *(4)* for yield and productivity, respectively. Other data pertaining to metabolic end-product distribution and operational parameters in connection with this series of subtraction refinement experiments are summarized in Table 3. For all formulations tested in this subtraction series, the values for both yield and productivity either met or surpassed the NREL goal, with the notable exception of the DS medium, which was included for purposes of comparison to illustrate the scope for improvement with respect to productivity. Since the ethanol yield is routinely based on the amount of sugar added to the medium, there exists the potential for yield inflation owing to the metabolism of nutrients other than added sugar when employing nutritionally rich, complex media such as LB *(37)*. Therefore, the use of the DS provides useful information regarding the contribution to ethanol production by the nonxylose components of the complex media. Our observations lend support to the claim of Grohmann et al. *(37)* regarding ethanol yield inflation by nonsugar components of the medium.

Apart from economic considerations, it is because of the reduction in growth rate and productivity that defined media are rarely used for the production of bulk chemicals *(25)*. When a single source of a complex nutritional supplement (e.g., YE or protein digest) is added to a chemically defined medium, the resulting formulation is termed a "semidefined" ("semisynthetic") medium *(25)*. Generally, the addition of relatively small amounts of organic (complex) nutrients supply growth factors and vitamins that can potentially significantly increase growth compared to

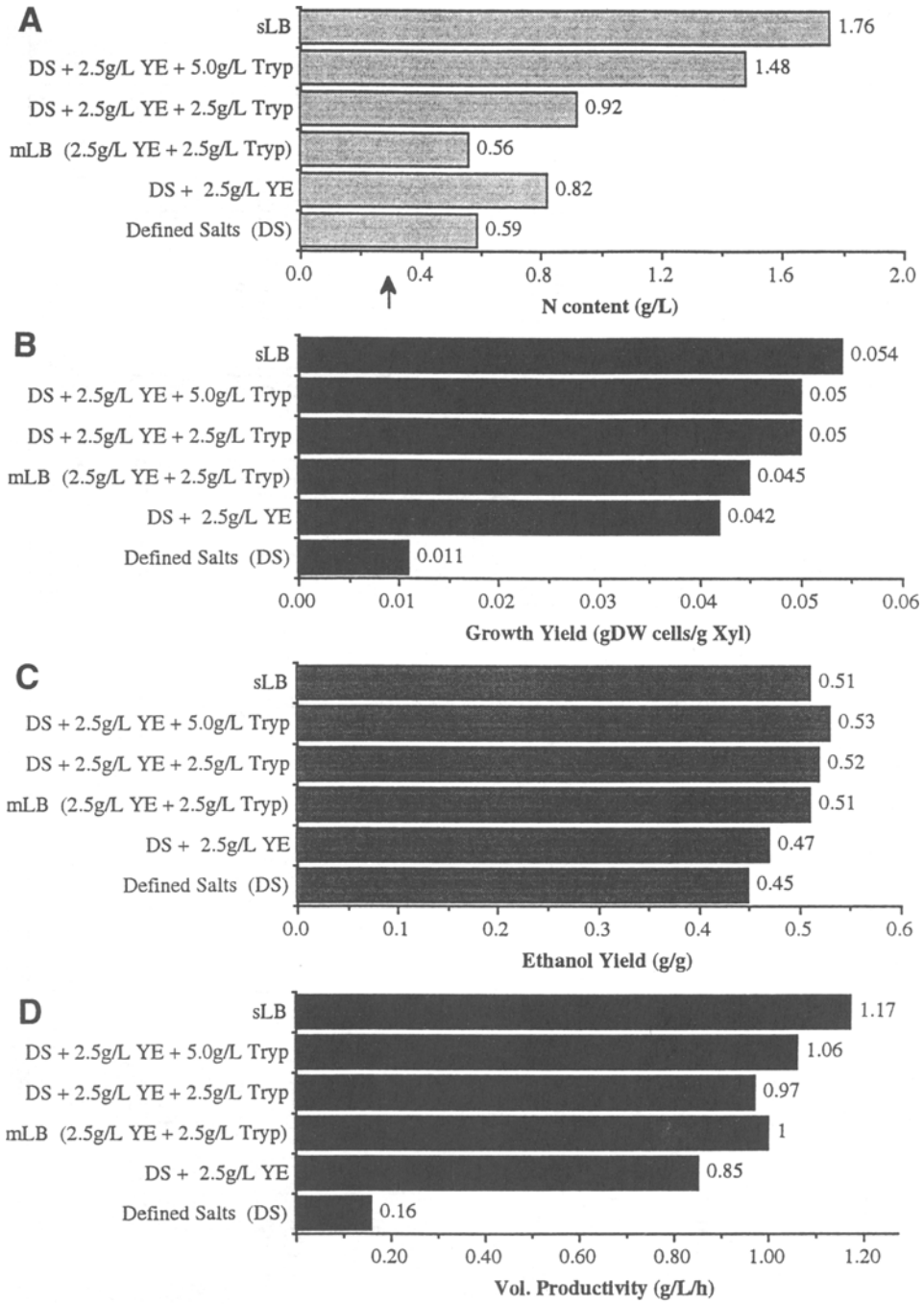


Fig. 1. The effect of subtraction refinements to sLB medium in terms of **(A)** total N content of the medium, **(B)** growth yield coefficient for xylose, **(C)** ethanol yield, and **(D)** volumetric productivity. All media contained about 4 wt% xylose. The arrow in A indicates the assumed minimal N concentration required to support 2 g dry wt cells/L. The arrows in C and D represent the NREL target values for these process parameters. Abbreviations regarding medium formulations are described in the text.

Table 3
Summary of End-Product Distribution and Operational Parameters

Expt. code	Medium, modification	Substrate [Xyl] g/L	Utilization Rate Q_s g/L·h	Utilization Rate max.Q_s g/L·h	Products [Cells] g dry wt/L	Products [EtOH] g/L	Products [Succ] mM	Products [Lac] mM	Products [Ac] mM	Yield cor.$Y_{p/s}$ g/g	pH	% Carbon recovery
B111a	sLB ("benchmark" reference)	42.4	1.93	2.29	2.28	20.52	31.2	0.0	13.2	0.51	7.0	113
B111b	DS + 2.5 YE + 5.0 Tryp	41.8	1.74	2.00	2.08	20.82	24.4	0.0	8.7	0.53	7.0	112
B133a	mLB	42.1	1.28	1.96	1.88	20.06	20.2	0.1	0.8	0.51	7.0	104
B146c	DS + 2.5 YE + 2.5 Tryp	41.9	0.87	2.00	2.11	20.58	22.3	0.1	15.3	0.52	7.0	112
B111c	DS + 2.5 YE	49.3	1.50	1.80	2.06	21.92	31.2	0.0	11.4	0.47	7.0	101
B110a	DS	40.5	0.34	0.36	0.44	17.00	15.9	0.0	18.0	0.45	7.0	92
B134c	DS + Vit + Glut	38.6	0.47	0.66	1.05	18.12	19.6	0.0	17.9	0.50	7.0	106
B135c	DS + Vit + AA	39.8	0.47	0.63	0.98	16.72	18.8	0.0	11.5	0.45	7.0	93
B135d	DS + Vit + AA$^+$	42.9	0.76	1.05	1.59	17.52	15.5	14.4	10.3	0.44	7.0	94
B133b	DS + Vit + 2.5 Tryp	42.2	1.03	1.35	1.82	18.97	22.9	3.5	11.9	0.48	6.3	103
B114a	TW + AP + 2.5 VYE	39.9	1.33	1.63	1.45	18.41	33.7	0.0	14.4	0.49	7.0	108
B114b	TW + AP + 1 Pan	41.7	1.30	1.64	1.39	18.00	33.4	0.0	14.2	0.47	7.0	101
B114c	TW + AP + 2.5 VYE + 1 Pan	40.4	1.44	1.66	1.58	17.35	38.9	0.0	15.9	0.46	7.0	104
B121b	TW + 37 mL CSL	36.3	1.45	1.96	1.26	19.56	25.3	0.0	14.3	0.57	7.0	122
B121c	TW + 18 mL CSL	35.9	1.33	1.64	1.10	17.83	46.8	0.0	16.6	0.53	7.0	105
B124a	TW + 18 mL CSL	44.7	1.32	1.57	1.21	18.34	49.6	0.0	16.8	0.44	7.0	100
Fermentations with 6% xylose												
B141a	sLB	62.7	2.02	2.40	2.98	26.71	25.9	0.0	14.5	0.46	7.0	96
B141b	TW + AP + 5.0 VYE	63.9	1.21	1.40	1.70	24.93	51.4	0.0	24.6	0.41	7.0	92
B141c	DS + 2X Vit + 0.25 Glut + AA$^+$	62.3	0.67	1.45	0.98	23.88	36.8	0.0	25.0	0.40	7.0	88
B141d	TW + 37 mL CSL	62.2	1.41	1.93	1.25	25.00	50.0	0.0	10.2	0.42	7.0	92

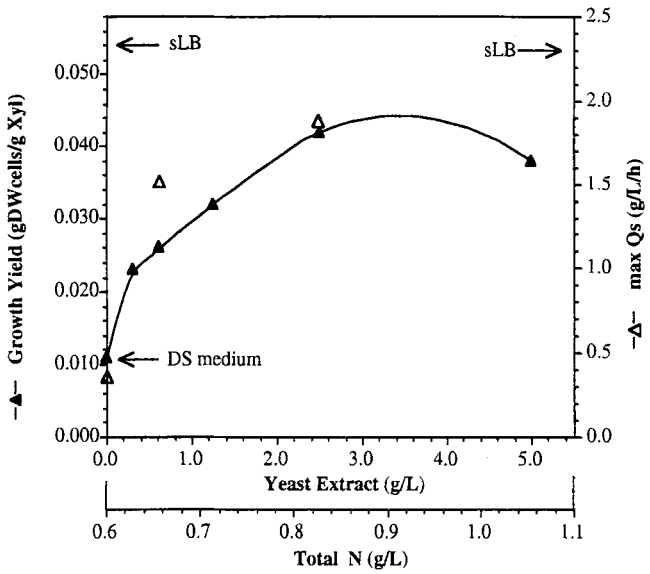


Fig. 2. The effect of incremental supplementation of the basic DS medium with YE. The basic medium contained about 4 wt% xylose and the pHE was controlled at 7.0. Symbols: filled triangles, growth yield coefficient based on xylose; open triangles, maximum rate of xylose utilization—*see* Materials and Methods for methods of determination. The arrows indicate the level of fermentation performance exhibited by recombinant *E. coli* (11303:pLOI297) in reference medium (sLB).

a fully defined medium. Figure 2 shows the effect of adding incremental amounts of Difco YE to the basic DS. In terms of both growth yield and the rate of xylose utilization, the optimal amount of YE appears to be about 2.5 g/L. Since the DS medium satisfies the N requirement (Table 2), the YE can be viewed primarily as a source of vitamins. Table 4 shows the vitamin composition of the DS medium supplemented with 2.5 g/L Difco YE. Based on calculated vitamin content of this medium (DS + 2.5 YE), we formulated a multicomponent synthetic vitamin supplement that, at an amendment rate of 5 mL/L, produces a medium closely approximating the YE-supplemented medium. The vitamin content of these media does not differ substantially from that for a defined *E. coli* medium described by Hernandez and Johnson *(38)* (Table 4). Tryptone is an enzymatic digest of casein, and similar components of complex media mean that such media contain a relatively complete complement of the amino acids. The supply of readily assimilable N in the form of amino acids in the culture medium results in an enhanced rate of growth because the necessity for biosynthesis from inorganic N and carbon metabolites derived from xylose catabolism is circumvented. The amounts of amino acids and vitamins in the complete defined medium used by others *(38,39)* have been based on the composition of bacteria *(27,40)*. In addition to vitamins, YE also supplies organic N, principally in the form of amino acids *(25)*. The content of YE with respect to aspartic and glutamic acids is 5.1 and 6.5 wt%, respectively. Figure 3 shows that supplementing DS with vitamins (5 mL/L stock solution) and 0.25 g/L Glut (DS + Vit + 2.5 Glut) produces a doubling in both the growth yield (Fig. 3B) and productivity (Fig. 3D). Supplementation of this medium with of a mixture containing an additional five different amino acids (DS + Vit + 2.5 Glut + AA) did

Table 4
Vitamin Content of Various Media Formulations

Vitamin/L	sLB	1/2 LB	mLB	DS + 2.5 YE	7.7 g/L CSL[a] 18 mL/L	This study, 5 mL stock/L	Ref.
Choline·Cl					26.9	5.0	
Inositol·$2H_2O$					46.1	7.5	309.0
Niacin (B_6)	1.46	0.728	0.725	0.700	0.640	0.750	0.323
Ca Pantothenate	NS				0.115	0.075	0.107
Pyridoxine·HCl	0.105	0.053	0.057	0.050	0.069	0.075	0.012
Riboflavin	0.096	0.048	0.048	0.048	0.046	0.050	0.103
Thiamine·HCl (B_1)	0.018	0.009	0.009	0.008	0.023	0.010	0.048
Biotin	0.009	0.004	0.004	0.007	0.002	0.005	0.013
Folic acid							0.103

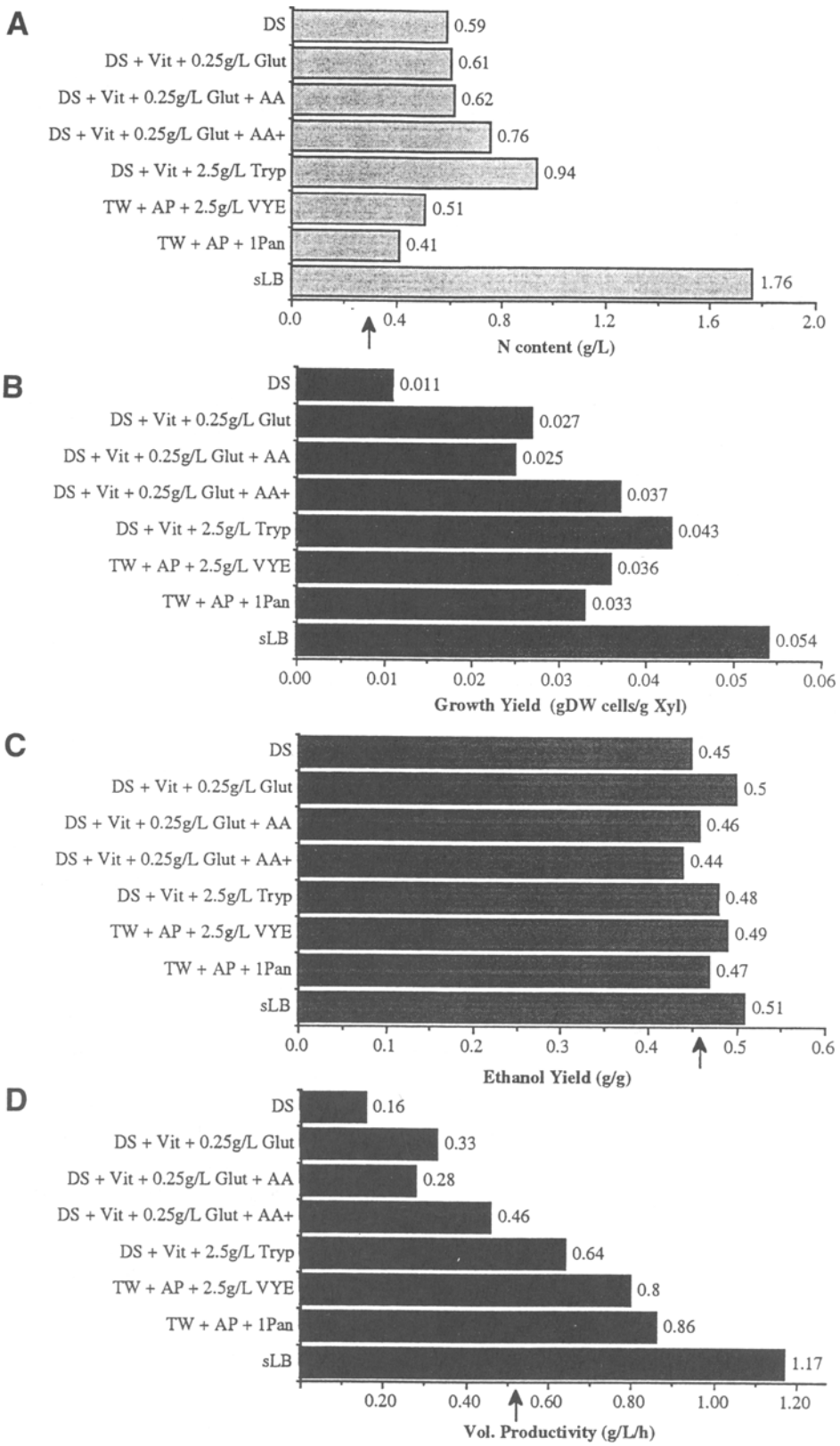
NS, not specified for BYE and Tryp(Difco).
Ref., Hernandez and Johnson *(38)*.
[a]Based on specifications for vitamin content of CSL (Corn Refiners Association Inc., Washington, DC).

not significantly produce an unexpected decrease in growth yield, ethanol yield, and productivity (Fig. 3). Further refinement through the addition of a full complement of amino acids as described in the formulation of Hernandez and Johnson *(38)* (DS + Vit + 2.5 Glut + AA^+) produced an improvement in growth (Fig. 3B) and productivity (Fig. 3D). The supply of amino acids in the culture medium bypasses the necessity for biosynthesis frown inorganic N and carbon metabolites derived from xylose catabolism, which results in an enhanced rate of growth.

Further incremental improvement in both growth and productivity was achieved by substitution of the complete amino acid supplement (including the Glut) by 2.5 g/L Tryp (DS + vit + 2.5 Tryp). Although this formulation produced a fermentation performance, in terms of productivity, that surpassed the NREL target value of 0.52 g/L·h, the productivity of 0.64 g/L·h was only about 50% of that achieved with sLB (Fig. 3D) and only 75% of the productivity achieved with basic DS medium that had been amended with 2.5 g/L YE (Fig. 2D). Interestingly, this formulation produced a disproportionate increase in the growth yield (Fig. 3B) relative to productivity (Fig. 3D) suggesting a possible negative impact on specific productivity.

All the formulations involving amendment of the basic DS medium contained sufficient N, P, and Mg to satisfy the requirements for the expected maximum cell yield of 2 g dry wt cells/L (Table 2). In a recently published technical and economical analysis of the production of fuel ethanol from lignocellulosic biomass

Fig. 3. *(Opposite page)* The effect of amendments to defined minimal medium in terms of **(A)** total N content of the medium, **(B)** growth yield coefficient for xylose, **(C)** ethanol yield, and **(D)** volumetric productivity. All media contained about 4 wt% xylose. The arrow in A indicates the assumed minimal N concentration required to support 2 g dry wt cells/L. The arrows in C and D represent the NREL target values for these process parameters. Abbreviations regarding medium formulations are described in the text.


A
DS
DS + Vit + 0.25g/L Glut
DS + Vit + 0.25g/L Glut + AA
DS + Vit + 0.25g/L Glut + AA+
DS + Vit + 2.5g/L Tryp
TW + AP + 2.5g/L VYE
TW + AP + 1Pan
sLB
0.59
0.61
0.62
0.76
0.94
0.51
0.41
1.76
0.0
0.4
0.8
1.2
1.6
2.0
N content (g/L)
B
DS
DS + Vit + 0.25g/L Glut
DS + Vit + 0.25g/L Glut + AA
DS + Vit + 0.25g/L Glut + AA+
DS + Vit + 2.5g/L Tryp
TW + AP + 2.5g/L VYE
TW + AP + 1Pan
sLB
0.011
0.027
0.025
0.037
0.043
0.036
0.033
0.054
0.00
0.01
0.02
0.03
0.04
0.05
0.06
Growth Yield (gDW cells/g Xyl)
C
DS
DS + Vit + 0.25g/L Glut
DS + Vit + 0.25g/L Glut + AA
DS + Vit + 0.25g/L Glut + AA+
DS + Vit + 2.5g/L Tryp
TW + AP + 2.5g/L VYE
TW + AP + 1Pan
sLB
0.45
0.5
0.46
0.44
0.48
0.49
0.47
0.51
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Ethanol Yield (g/g)
D
DS
DS + Vit + 0.25g/L Glut
DS + Vit + 0.25g/L Glut + AA
DS + Vit + 0.25g/L Glut + AA+
DS + Vit + 2.5g/L Tryp
TW + AP + 2.5g/L VYE
TW + AP + 1Pan
sLB
0.16
0.33
0.28
0.46
0.64
0.8
0.86
1.17
0.20
0.40
0.60
0.80
1.00
1.20
Vol. Productivity (g/L/h)

(willow) using recombinant *E. coli* KO11, von Sivers et al. *(41)* suggested that in large-scale fermentations the cell mass would increase from 1 to 3 g dry wt cells/L during each fermentation and that the nutritional requirements would be satisfied by a medium containing 0.4 g N, 0.2 g P, and 0.02 g Mg/L. They did not recommend a specific recipe or source for these elements. These estimates of von Sivers et al. *(41)* are based on measurements of the stoichiometry of these elements in cell mass and therefore are similar to the ratios shown in Table 2.

Cost-Effective Nutrient Supplementation for Large-Scale Fermentations

Utilizing Less-Expensive Sources of Yeast Extract and Tryptone

In the light of nutritional information gained through supplementation of the basic defined minimal medium and subtraction refinements to the chemically rich complex reference medium, the essence of cost reduction involves the substitution of expensive lab-grade ingredients with nutritionally equivalent, less costly, commercially available supplements such that the fermentation performance of the biocatalyst is not compromised as a consequence of the substitution or reformulation. With YE and Tryp costing about $100 and $83/kg, respectively, the cost of the reference LB medium ($1/L) is clearly not economical in terms of the production of bulk chemicals. In a series of flask fermentations with buffered media, it was determined that tap water could be used effectively as a source of trace elements and that 1.4 g/L ammonium phosphate satisfied the requirement for N (results not shown). Two such TW +AP formulations were assessed: one contained 2.5 g/L Veeprex (a commercial YE product costing about $8/kg) and the other contained 1 g/L Pancase S (a commercial casein digest product costing about $27/kg). These formulations met the requirements for N, P, and Mg (Fig. 3A and Table 2) and provided an improved fermentation performance over the formulations involving DS supplementation (Fig. 3). Although these TW formulations surpassed the NREL targets for yield and productivity (Fig. 3 C,D), the productivity was only about 70% compared to the "benchmark" value that was established with sLB (Fig. 3D).

Corn Steep Liquor as a Single-Source Nutrient Supplement

CSL is a byproduct of corn wet-milling and is marketed primarily as an animal feed supplement with a current selling price of about $50/t. CSL contains a rich complement of important nutrients (including vitamins—Table 4) to support robust microbial growth and fermentation *(42)*. It was first used as a nutrient source in the 1940s in the development of large-scale penicillin fermentations and continues to be used extensively today in diverse industrial fermentation processes. The process (light) steep water is concentrated by evaporation to about 40–50% solids to produce heavy steep water or CSL *(42)*. The protein content (on average about 40–50%) is estimated from the determination of the total Kjeldahl N (N × 6.25) and, hence, N content quoted in Table 2 does not truly represent the assimilable N content.

The results of a series of pH-controlled batch fermentations designed to determine the optimal amount of CSL as a single-source nutrient supplement are shown in Fig. 4; the relevant data relating to metabolic end product distribution, carbon recovery, and other operational parameters are summarized in Table 3. At

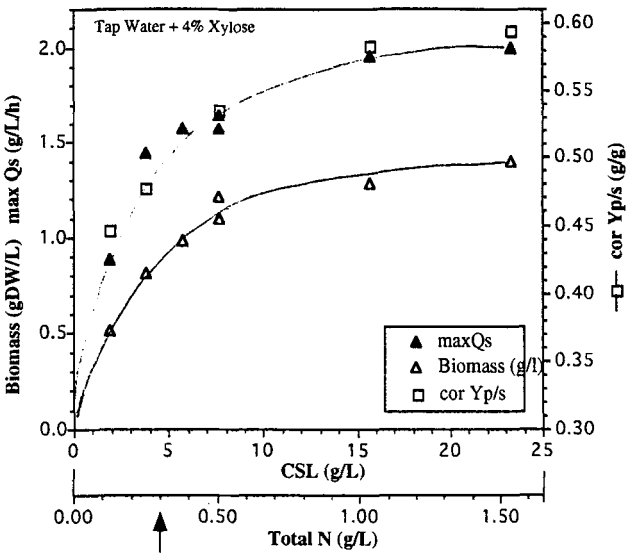


Fig. 4. Comparative fermentation performance achieved by recombinant *E. coli* in a medium consisting of the effect of incremental supplementation of 4 wt% xylose with CSL. The xylose was prepared with tap water. The pH was controlled at 7.0. Symbols: open triangles, maximum cell density (g dry wt cells/L); filled triangles, maximum rate of xylose utilization; open squares, ethanol yield based on xylose consumption and corrected for dilution by alkali added during fermentation. The arrow indicates the assumed minimal N concentration required to support 2 g dry wt cells/L.

4% xylose, there is only a marginal improvement in both growth yield and productivity at supplementation levels >18 mL CSL/L (equivalent to 7.7 g/L) (Fig. 4). Owing to the contribution of nonxylose components of CSL, the ethanol yield at the higher amendments exceeded the theoretical maximum value of 0.51 g/g xylose (Fig. 4). The productivity (estimated from $maxQ_s$) achieved with optimal levels of CSL corresponded to about 90% achieved with the reference sLB medium.

Based on the N content of *E. coli*, others have estimated that it would require 7 g CSL/L to achieve a cell density of 3 g dry wt cells/L *(43)*. Amartey and Jeffries *(44)* used CSL to replace YE and other nutrients in xylose fermentations by *Pichia stipitis*. Beall et al. *(45)* supplemented a corn hulls acid hydrolysate with 20 mL CSL/L (equivalent to 9 g/L at 45% solids). It should be noted in comparing our results to those of Beall et al. *(45)* that all our media were sterilized by autoclaving, whereas theirs were filter-sterilized. It is not known to what extent the content of vitamins and other growth factors might be affected by the sustained heat treatment of autoclaving. Nevertheless, in general, our findings appear to substantiate Ingram's recommendation regarding the sufficiency of this amount of CSL in supporting good growth and xylose fermentation by recombinant *E. coli*.

Unfortunately, there are disadvantages associated with the use of CSL as a nutritional supplement. Apart from the fact that the composition is not invariant, CSL does not have an indefinite shelf life. The steeping process in corn wet-milling involves addition of SO_2 to the steep water, which can yield substances in the CSL that are potentially inhibitory to bacterial growth. Fortunately, most of the sulfur dioxide is removed during the evaporation of the steep water in the production of

heavy steep water (CSL at about 40–50% solids). However, lactic acid bacteria are known to be prevalent in CSL, and they are not totally eradicated during the evaporation stage. CSL contains lactic acid and the concentration of this acid increases with storage even at refrigeration temperatures. Propagation of bacteria can be expected to affect the potential nutritional value of the CSL. It is not known how lactic acid (or other byproducts of LAB metabolism) might affect the fermentation performance of the biocatalyst.

Fermentations with Selected Media Formulations (6% Xylose)

The economic analysis of Hinman et al. *(5)* with respect to the impact of hemicellulose utilization in the conversion of lignocellulosic feedstocks to fuel ethanol was based on 6% xylose, "which seems to be the highest concentration that can reasonably be obtained using dilute-acid pretreatment without a xylose concentrating step." Therefore, in order to test the ability of the recombinant *E. coli* to achieve the target final ethanol concentration of 25 g/L, a series of batch fermentations were conducted with selected media at the upper limit of xylose concentration to be anticipated in dilute-acid prehydrolysates (approx 6 wt%).

Under a condition of xylose-limited growth, a medium containing 60 g/L xylose would be expected to produce a maxium cell yield of about 3 g dry wt cells/L *(33)*. Therefore, for none of these major essential elements to be growth-limiting, the 6% xylose medium should contain (at a minimal level) 0.45 g N/L, 0.045 g P/L, and 0.005 g Mg/L. Table 2 compares the composition of the selected media with respect to N, P, and Mg. Clearly, all the selected media formulations contain an excess of these estimated threshold levels of N, P, and Mg.

The results of this series of batch fermentations are represented in Fig. 5 and Table 3. Only in the case of the reference medium (sLB) was the ethanol yield target value of 0.46 g/g achieved (Fig. 5D). However, the lower than usual value for carbon recovery in these experiments is perhaps a reflection of the underestimation of the ethanol produced in these systems (Table 3). All media formulations resulted in a final ethanol concentration that met the target value of 25 g/L (Fig. 5C). Likewise, with all 6% xylose media formulations, the productivity goal of 0.52 g/L·h was surpassed (Fig. 5D).

In the economic analysis of Grethlein and Dill *(43)*, which was based on a batch process using recombinant *E. coli* in a biomass hydrolysate supplemented with CSL, it was assumed that 40 g ethanol could be derived from 80 g sugars (equivalent to a process yield of 0.50 g/g or 98% conversion efficiency) and that the fermentation would be complete in 48 h (equivalent to a Q_s value of 1.67 or a volumetric productivity of 0.84 g/L·h). In the experiment with the CSL-based medium, the yield (corrected for dilution by alkali) was 0.43 g/g (83.4% conversion efficiency) (Fig. 5B) and the productivity was 0.82 g/L·h (Fig. 5D). Based on the final cell concentration of 1.25 g dry wt cells/L (Table 3), this is equivalent to specific productivity of 0.66 g/g·h. In a recent economic assessment of biomass prehydrolysate fermentation using recombinant *E. coli* KO11, von Sivers et al. *(41)* used a value for specific productivity that was half the value calculated for the fermentation with CSL supplementation.

Economic Impact of Nutrient Supplementation on Cost of Fuel Ethanol from Biomass

With respect to the cost effectiveness of the various media formulations that were examined in this study, nothing can compete with the CSL-based medium. At

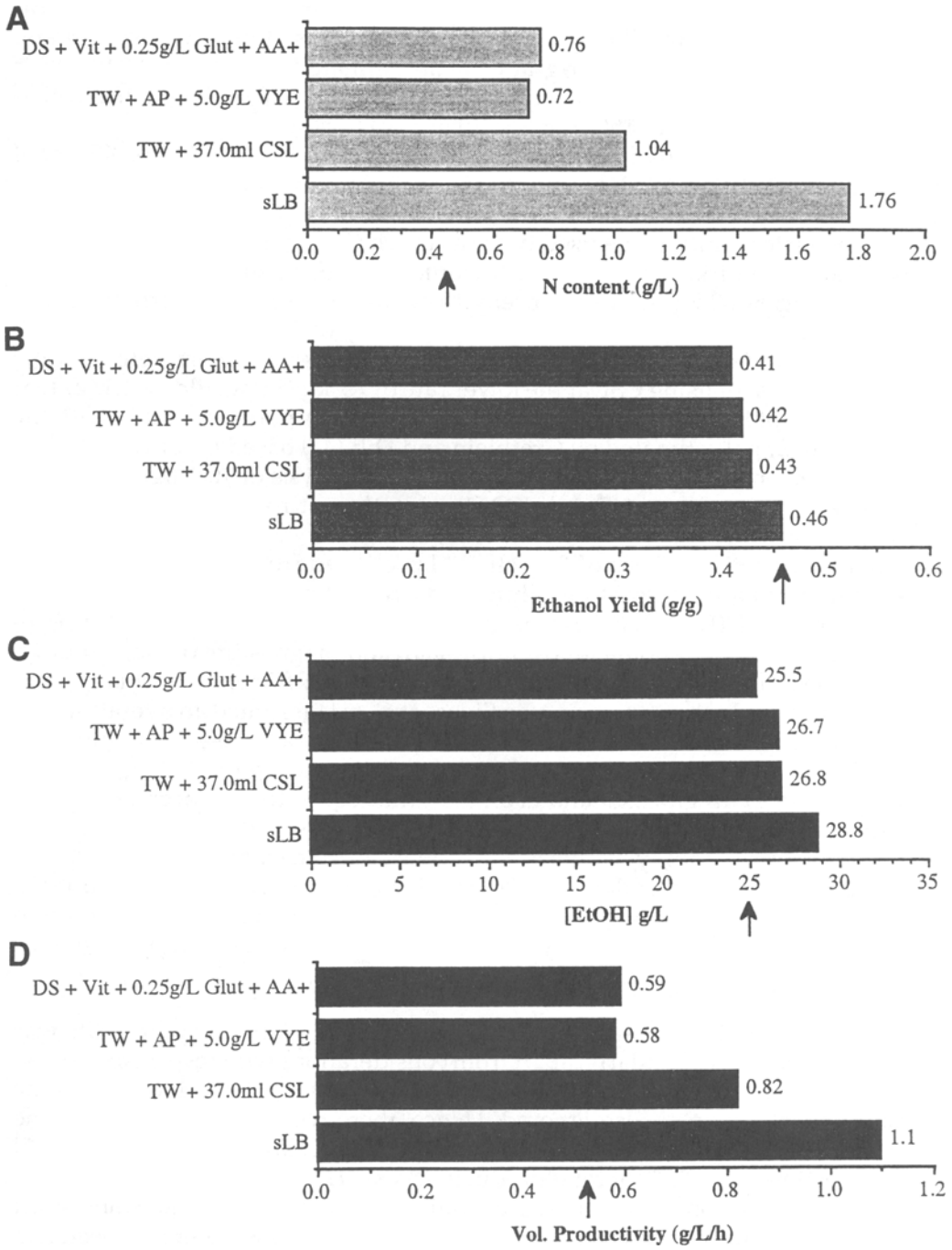


Fig. 5. Fermentation with selected media at 6 wt% xylose in terms of **(A)** total N content of the medium, **(B)** ethanol yield, **(C)** final ethanol concentration, and **(D)** volumetric productivity. The arrow in A indicates the assumed minimal N concentration required to support 3 g dry wt cells/L. The arrows in B, C, and D represent the NREL target values for these process parameters. Abbreviations regarding medium formulations are described in the text.

a cost of about \$1/L, the laboratory reference medium (sLB) is clearly well off the economic scale of feasibility. Even the medium based on VYE (\$8/kg) and ammonium phosphate (fertilizer-grade), at an estimated cost of about \$40/m^3, is well beyond economic feasibility (equivalent to cost of \$4.10/gal denatured ethanol). However, at a cost of \$50/t of CSL (5.5¢/kg) (dry wt basis), the cost of CSL supplementation of prehydrolysate at a rate equivalent to 7.7 g/L is \$0.42/m^3 of fermentation medium. At a level of 4% xylose, this amount of CSL seems saturating in terms of growth, cell concentration, and ethanol productivity. To ensure that CSL was not limiting, we used twice as much CSL in the fermentation at 6% xylose, but it is not known if less CSL would have been sufficient. Based on a xylose loading of 60 kg/m^3 in the prehydrolysate feed to the C_5 fermenter and assuming a xylose-to-ethanol conversion efficiency of 90% of theoretical maximum (equivalent to the NREL yield goal 0.46 g/g) *(4)* and complete product recovery, the cost of using CSL at the lower rate of 7.7 kg/m^3 would be 1.13¢/L or \$0.043/US gal of E95 fuel ethanol. This cost estimate compares favorably with the value of \$0.042/gal estimated by Grethlein and Dill *(43)* based on CSL as a nutritional supplement for a process using enzymatic hydrolysis of 10% lignocellulosic biomass and recombinant *E. coli* KO11 operating at near 100% conversion efficiency.

In an economic analysis of fuel ethanol produced from lignocellulosic biomass (willow) in a process using dilute-acid pretreatment and fermentation by recombinant *E. coli* (KO11), von Sivers et al. *(41)* based the cost of nutrient supplementation solely on ammonia, phosphoric acid, and magnesium oxide, although there has been no experimental confirmation of the sufficiency of this type of supplementation. In their analysis, von Sivers et al. *(41)* assumed an overall sugar-to-ethanol conversion efficiency of 92% (94% efficiency of xylose-to-ethanol conversion by recombinant *E. coli* KO11) with a xylose loading or 42.7 kg/m^3 (also containing 18 kg/m^3 glucose) and estimated the cost of their inorganic supplementation to be \$192,000/yr (based on a plant producing at total of 6.9 million liters of E95 fuel ethanol/yr). This is equivalent to a nutrient supplement cost of 2.78¢/L or \$0.106/US gal of denatured ethanol. von Sivers et al. *(41)* also proposed that the pure culture (inoculum preparation) plant associated with this operation would use prehydrolysate similarly supplemented with these inorganic elements.

Other Relevant Economic Considerations

In this study, the potential nutritional value of the biomass hydrolysate was not taken into account. Similarly, apart from considerations with respect to controlling the pH at 7.0 to minimize the effect of acetic acid, the potential toxicity of the biomass hydrolysate was also ignored. Hence, there has been no regard for the potential cost relating to detoxification. Detoxification costs can be considerable *(4)* and were estimated at 22% of total production cost *(41)*.

In an industrial operation, there is a requirement for make-up water, and apart from the contribution of the biomass hydrolysate, this process water is expected to be a good source of trace elements and other essential minerals. This study examined the nutritional requirements of the biocatalyst within the context of a batch fermentation process, but did not examine the effect of multiple sequential batch fermentations (cycles) in which a portion of the stillage (usually at 8–10% by volume) is used for the purpose of seeding the next batch (back set). This practice has implications in terms of the amounts of supplements required. Furthermore, it is

anticipated that in continuous fermentation processes, there is an opportunity for nutrient cost reduction through recycling of cells and stillage.

Finally, this study did not address the issue of the stability of the genetically engineered biocatalyst. Even if antibiotics are required only for the generation of inocula (so-called pure culture operation), the economic impact will depend in a large part on the required frequency of seeding with fresh (pure) culture. In their recent cost analysis based on the use of recombinant *E. coli* KO11, von Sivers et al. *(41)* estimated this requirement to be only three times a year. However, there was no evidence regarding the stability of this organism to validate this assumption, and our recent findings on this subject cast doubt on the presumed stability of this chromosomally integrated recombinant *(30)*. Setting aside any possible regulatory obstacles in terms of employing a genetically engineered biocatalyst, it is estimated the cost of antibiotic ($55/kg) for the purpose of ensuring and maintaining high performance of the biocatalyst is as important a consideration as the cost effectiveness of nutrient supplementation.

ACKNOWLEDGMENTS

This research was part of Phase 1 of a Subcontract AAP-4-11195-03 from the National Renewable Energy Laboratory. We are grateful to Lonnie Ingram for the gift of the recombinant *E. coli* culture.

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