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Corn Steep Liquor as a Cost-Effective Nutrition Adjunct in High-Performance *Zymomonas* Ethanol Fermentations

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ABSTRACT

The ethanologenic bacterium *Zymomonas mobilis* has been demonstrated to possess several fermentation performance characteristics that are superior to yeast. In a recent survey conducted by the National Renewable Energy Laboratory (NREL), *Zymomonas* was selected as the most promising host for improvement by genetic engineering directed to pentose metabolism for the production of ethanol from lignocellulosic biomass and wastes. Minimization of costs associated with nutritional supplements and seed production is essential for economic large-scale production of fuel ethanol. Corn steep liquor (CSL) is a byproduct of corn wet-milling and has been used as a fermentation nutrient supplement in several different fermentations. This study employed pH-controlled batch fermenters to compare the growth and fermentation performance of *Z. mobilis* in glucose media with whole and clarified corn steep liquor as sole nutrient source, and to determine minimal amounts of CSL required to sustain high-performance fermentation.

It was concluded that CSL can be used as a cost-effective single-source nutrition adjunct for *Zymomonas* fermentations. Supplementation with inorganic nitrogen significantly reduced the requirement for CSL. Depending on the type of process and mode of operation, there can be a significant contribution of nutrients from the seed culture, and this would also reduce the requirement for CSL. Removal of the insolubles (40% of the total solids) from CSL did not detract significantly from its nutritional effectiveness. On an equal-volume basis, clarified CSL was 1.33 times more "effective" (in terms of cell mass yield and fermentation time) than whole CSL. For fermentations at sugar loading of >5% (w/v), the

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recommended level of supplementation with clarified CSL is 1.0% (v/v). Based on CSL at US $50/t, the cost associated with using clarified CSL at 1.0% (v/v) is 88¢/1000 L of medium and 5.3¢/gal of undenatured ethanol for fermentation of 10% (w/v) glucose. This cost compares favorably to estimates for using inorganic nutrients. The cost impact is reduced to 3.1¢/gal if there is a byproduct credit for selling the insolubles as animal feed at a price of about US $100/t. Therefore, the disposition of the CSL insolubles can significantly impact the calculations of cost associated with the use of CSL as a nutritional adjunct in large-scale fermentations.

Index Entries: *Zymomonas*; clarified corn steep liquor; whole CSL; nutrition; ethanol; economic impact; cell yield; high-performance fermentation; insolubles; defined medium.

INTRODUCTION

In North America, the increasing practice of a legislated requirement for oxygenate additives in gasoline offers a growth opportunity for the fuel ethanol industry. At the present time, this industry relies primarily on the fermentation of glucose derived from corn or cereal grain starch, and to a much lesser extent, fructose (sucrose) from cane and beet sugar *(1,2)*. For the most part, the technology used in the large-scale production of fermentation fuel ethanol is the same as that currently practiced in the beverage alcohol industry where yeast is used to convert sugar to ethanol *(1,2)*.

For the fuel ethanol industry to expand and remain economically viable, there is a requirement for alternative sources of fermentable carbohydrates *(3,4)*, and the "yeast monopoly" *(1,2)* is now being seriously challenged by other ethanologenic biocatalysts that have been either selected or engineered for their improved fermentation efficiency *(5,6)*. The bacterium *Zymomonas* (for reviews, *see 7,8)* has proven fermentation performance characteristics that are superior to yeast *(9–14)*, and this biocatalyst has the potential to "revolutionize the industry" *(15)*. In a survey of several microorganisms that was recently conducted by the National Renewable Energy Laboratory (NREL), *Zymomonas* was selected as the most promising host for improvement by genetic engineering directed to pentose metabolism *(16,17)*.

The biological approach to process improvement is directed toward the performance characteristics of the biocatalyst *(18)*, and although major developments are usually credited to the selection or engineering of a superior strain, other parameters, such as the nutritional and physical environment to which the biocatalyst will be exposed, are also known to have a significant potential influence on yield and productivity both with respect to growth and fermentation *(19)*. Minimization of costs associated with nutritional supplements and seed production is essential for economic large-scale production of fuel ethanol *(20)*.

Most *Zymomonas* strains are known to be auxotrophic for the vitamin pantothenic acid *(7,21–24)*, and although there are several reports in the literature of defined and minimal media formulations commensurate with high-performance fermentation by *Zymomonas (11,18,25–28)*, relatively little is understood concerning the specific influence on growth and fermentation of the individual medium components. Furthermore, these defined media are not useful in an industrial context owing to the prohibitive cost of the vitamin supplements.

Corn steep liquor (CSL) is a byproduct of corn wet-milling, and contains a rich complement of important nutrients to support robust microbial growth and fermentation *(29)*. 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 (LSW) is concentrated about 10-fold by evaporation to 45–55% solids to produce heavy steep water (HSW) or CSL. CSL is sold into the animal feed market and has a protein value judged to be equivalent to gluten feed selling for US $100/t. Since on average CSL is about 50% dry substance *(29)*, the selling price is US $50/t. The protein content is estimated from the determination of the total Kjeldahl nitrogen *(29)*. As a fermentation medium supplement, CSL can be viewed either as a complete source of nutrients or as a source of vitamins and other trace elements (growth factors) *(19,30)*. Since *Zymomonas* can assimilate inorganic nitrogen *(18)*, supplementation with inorganic nitrogen potentially could significantly reduce the level of CSL required to support growth and fermentation.

Previous work conducted in our lab *(12,15,31,32)* as well as that of others *(33,34)* has showed that LSW and HSW from corn wet-milling were effective nutritional supplements for *Zymomonas* fermentations. CSL has also been investigated in terms of its equivalence to yeast extract as a complex nutritional supplement for xylose-fermenting yeasts *(35)* and used in the development of a yeast-based simultaneous saccharification and fermentation biomass-to-ethanol process *(36)*. CSL has also been used in ag-waste and wood biomass-processes that propose to use recombinant *Escherichia coli (37–40)*.

The purpose of this study was fourfold:

1. To compare the fermentation performance of a wild-type strain of *Zymomonas mobilis* CP4 in media with whole and clarified CSL as sole nutrient source;
2. To determine minimal amounts of CSL required to sustain high-performance fermentation;
3. To examine the potential for reducing the amount of CSL through supplementation with inorganic nitrogen; and
4. To estimate the economic impact of using CSL on the cost on producing fuel ethanol.

MATERIALS AND METHODS

Organisms

The wild-type strain *Z. mobilis* CP4 was received from M. Zhang (National Renewable Energy Laboratory, Golden, CO). Cultures, grown from single-colony isolates on glucose agar medium, were stored at –70°C in a nutrient-rich complex medium supplemented with antifreeze (glycerol at 15 mL/dL).

Fermentation Media

The chemical composition of the different media used in this study is given in Table 1. For comparative purposes, the reference medium was a nutritionally rich, complex medium containing 3% (w/v) yeast extract (Difco Laboratories, Detroit, MI) and was designated as "ZM1" (Table 1). In the absence of yeast extract, the defined salts medium was designated as "DS" medium, and it was supplemented with the vitamins D-pantothenic acid (hemi-calcium salt, 1.0 mg/L) and biotin (1.0 mg/L) (Sigma Chemical, St. Louis, MO) (Table 1). D-Glucose was added (as specified) as the sole carbon (energy) source. The media were sterilized by autoclaving with the stock glucose solution being autoclaved separately to minimize browning.

Two samples of CSL were obtained from NACAN Products (Collingwood, Ontario, Canada) on separate occasions and stored in a refrigerator at 4°C. The "CSL medium" (Table 1) consisted of autoclaved tap water (TW) supplemented with either whole (wCSL) or centrifugally clarified corn steep liquor (cCSL), which was added at the time of inoculation. Following centrifugation (10,000*g* for 10 min), the packed sediment represented 25% of the total volume.

Fermentation Equipment

Batch fermentations were conducted in 1- or 2-L stirred-tank bioreactors (STR) fitted with pH control *(30)*. The temperature was kept constant at 30°C. A bench-top chemostat with a working volume of 350 mL (Bioflo C-30, New Brunswick Scientific, Edison, NJ) was used to generate glucose-limited continuous cultures as described previously *(11)*.

Methods of Preculture and Inoculation Procedures

A 1-mL aliquot of a glycerol-preserved culture was removed from cold storage and transferred aseptically to a 125-mL screw-cap Erlenmyer flask containing about 100 mL of either ZM1 medium or 2% (v/v) cCSL medium with 2% (w/v) glucose. The CSL medium was supplemented with KH_2PO_4 (20 g/L), and the initial pH adjusted to 6.0. Seed flask cultures were grown statically overnight in an incubator (30°). In

Table 1
Fermentation Media Formulations[a]

Ingredient (g)	Medium Designation		
	ZM1	DS	CSL
D-Glucose	var	var	var
Yeast Extract (Difco)	3.00	0	0
NH_4Cl	0.81	1.6	var
cCSL (mL)	0	0	var
KH_2PO_4	3.48	3.48	0*
$MgSO_4$	0.49	0.49	0
$FeSO_4.7H_2O$	0.01	0.01	0
Citric acid	0.21	0.21	0
Ca Pantothenate	0	0.001	0
Biotin	0	0.001	0
Distilled water (L)	1	1	-
Tap water (L)	-	-	1

[a]var = amount variable (as specified for each fermentation); DS = defined salts medium; cCSL = clarified CSL.

*KH_2PO_4 (2 g/L) added for pH buffering in seed flask cultures.

experiments where a chemostat culture was used as seed, the effluent was collected on ice overnight.

STR batch fermentations were inoculated by transferring approx 10% (v/v) of the overnight flask culture directly to the medium in the bioreactor. The initial cell density was monitored spectrophotometrically to give an OD_{600} in the range 0.1–0.2, corresponding to 30–50 mg dry cell mass (DCM)/L. An alternative inoculation procedure was developed that minimized the potential for transfer of nutrients from the preculture medium during inoculation. This method involved a typical centrifugal harvesting/washing procedure. Following overnight growth at 30°C, the culture was centrifuged at 10,000*g* for 10 min (Sorvall RC2B centrifuge). The cell pellet was resuspended in 0.1% (w/v) peptone (Difco) water, agitated to achieve uniformity and used to inoculate the STR at the desired initial cell density (OD). This procedure was designated as the "concentrated cell inoculation" (CCI) method.

Analytical Procedures

Growth was measured turbidometrically at 600 nm (1-cm lightpath) (Unicam spectrophotometer, model SP1800). In all cases, the blank cuvet contained distilled water. DCM was determined by microfiltration of an

aliquot of culture, followed by washing and drying of the filter to constant weight under an infrared heat lamp. Compositional analyses of fermentation media and cell-free spent media were accomplished by HPLC as described previously *(30)*.

RESULTS AND DISCUSSION

In this study, we adopted the strategy commonly practiced in microbial physiology for investigating microbial nutritional requirements through culture media formulation. Microorganisms exhibit growth and metabolism (fermentation) optima with respect to both physical and chemical environmental factors. A prerequisite to growth is that the medium supply the elements of carbon, nitrogen, phosphorous, and sulfur that, in addition to hydrogen and oxygen, are the major components of all biomolecules. In addition to sodium, potassium, magnesium, calcium, and iron, certain minerals are required in relatively much smaller amounts ("trace elements"). There is a low-level requirement for metals, such as manganese, copper, cobalt, molybdenum, zinc, and so forth, that act as enzyme co-factors. In addition, there can be certain other essential elements that, apart from the known "vitamins," are referred to collectively as "growth factors." The elemental composition of the organism reflects the mass ratio requirements of these various nutritional elements in the culture medium. However, in this context, it is important to bear in mind that cellular composition can be influenced by the chemical nature of the growth environment. Accordingly, our approach to formulating a cost-effective medium with minimal levels of CSL involved a balancing of the nitrogen content of the medium with the nitrogen requirement of the anticipated cell mass concentration. A prerequisite to this approach to medium formulation is a knowledge of the nitrogen content of both the nutritional supplement (in this case CSL) and the *Zymomonas* cell mass.

In a separate study that was reported at this meeting, we were interested in optimizing seed production for a biomass-to-ethanol process that proposes to use a recombinant *Zymomonas (41)*. For the purpose of seed production, the objective is to maximize cell density, and in a pH-controlled batch culture, this can be accomplished using a semisynthetic medium, such as ZM1 (Table 1), where both yeast extract (YE) and inorganic nitrogen (NH_4Cl) act as sources of assimilable nitrogen (Fig. 1). In the plot of cell mass vs glucose concentration (Fig. 1), the slope of the tangent provides an estimation of the growth yield with respect to carbon (energy) source, which under these conditions, is 0.036 g DCM/g glucose. With the pH controlled at 5.0, a maximum cell mass concentration of 2.25 g DCM/L is achieved at a glucose level of about 65 g/L, with very little further increase in cell density at higher sugar loading (Fig. 1). The growth yield is known to be affected by pH *(42,43)*, and we observed that the maximum cell mass concentration can be increased about 10% to

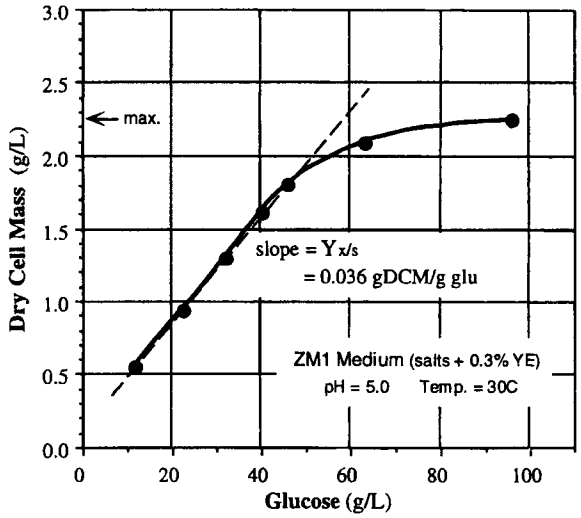

Fig. 1. Production of *Zymomonas* cell mass as a function of glucose concentration. Batch cultures of *Z. mobilis* CP4 were conducted in a semisynthetic medium (ZM1) containing glucose as sole carbon source (range approx 10–100 g/L). The pH and temperature were kept constant at 5.0 and 30°C, respectively. The *y*-axis arrow shows the observed maximum cell density of 2.25 g DCM/L.

about 2.6 g DCM/L by increasing the pH control set point from 5.0–6.0 (results not shown).

A Kjeldahl assay for total nitrogen (N) of the cell mass of *Z. mobilis* CP4 resulted in an average value of 13.6% dry basis (% db). This is equivalent to a growth yield with respect to N of 7.35 g DCM/g atom N, which agrees very closely with the value of 7.1 reported previously by Lawford and Stevnsborg *(11)* for a nitrogen-limited steady-state chemostat culture of *Z. mobilis* ATCC 29191 using a defined salts medium with ammonium chloride as the sole source of assimilable nitrogen. According to the concept of nitrogen balancing, the minimum level of N in the medium required to satisfy the requirement for the synthesis of 2.6 g DCM/L would be 0.354 g of assimilable N/L. This requirement could be supplied by 3.86 g/L of Bacto yeast extract (Difco) (total N = 9.18% db) or alternatively by a source of inorganic nitrogen, such as ammonium chloride, at a level of 1.35 g/L. It should be noted that these levels of N supplementation by yeast extract or NH_4Cl represent the minimum amounts required to satisfy the N requirement for a cell concentration of 2.6 g DCM/L, and assume an equivalence between total N and assimilable N.

This study is an extension of previous work that involved testing the fermentation performance response of a recombinant ethanologenic *E. coli* with a view toward designing a cost-effective, nutritionally lean medium for large-scale cellulosic ethanol production *(30)*. However, whereas previously we relied on CSL product specifications given to us by the supplier, in this study, we analyzed the two samples of CSL that were

Table 2
Composition of CSL[a]

Composition	cCSL	cCSL	wCSL
Batch No.	1	2	2
Density	1.12	1.13	1.20
Percent Solids (w/w)	46.1	41.4	44.2
Insolubles (% db)	ND	ND	40.0
Ash (% db)	15.8	ND	ND
Protein (% db) Total Kjeldahl N x 6.25	45.8	44.4	42.5
Carbohydrate (% db) (by difference)	16.5	ND	ND
Glucose (% db)	ND	0.19	ND
Lactic acid (% db)	ND	18.8	ND
Volumetric Total N (gN/mL)	0.0378	0.0333	0.0364

[a]CSL was from Nacan Products Ltd. (Collingwood, ON, Canada). wCSL = whole CSL; cCSL = centrifugally clarified CSL; % db = percent dry basis; ND = not determined.

provided to us at different times from a local corn wet-milling operation. The results of our compositional analysis of CSL are summarized in Table 2. Another distinguishing feature of this study was the use of cCSL. The high turbidity of the cell-free medium that is caused by the solids (insolubles) of the CSL supplement compromises the turbidometric measurement of growth, and the use of cCSL overcame this problem. The wCSL contained 44.2% solids (i.e., 44.2% by weight is dry substance) and 40% (db) insolubles that could be removed by centrifugation (Table 2)—the packed volume of the insoluble matter was 25%. In experiments designed to test for minimal levels of nutritional supplementation by a complex adjunct, such as CSL, it is important to restrict (minimize) the transfer of nutrients during inoculation. For this reason, in this work, we adopted a procedure of using centrifugally harvested cells as inoculum (*see* Materials and Methods).

Figure 2 shows typical time-courses for growth and glucose utilization by *Z. mobilis* CP4 in a medium consisting solely of TW and 4% (w/v) glucose that was amended with different amounts of cCSL over the range 4–20 mL/L. The pH was controlled at 5.0, and the temperature was 30°C. For comparative purposes, ZM1 medium (4% glucose) was used as the "control"

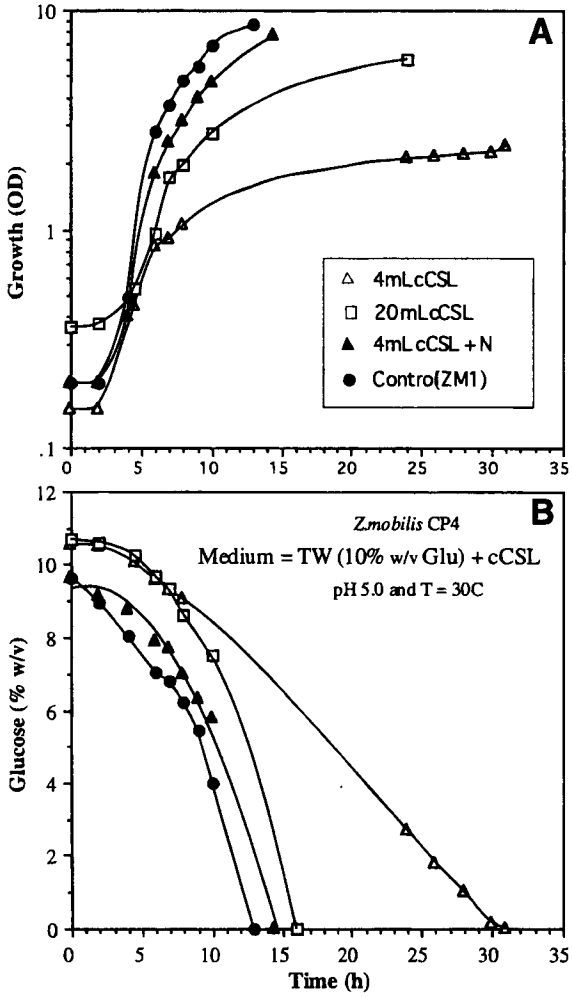

Fig. 2. Time-course of batch fermentations with Z. *mobilis* CP4. **(A)** Growth and **(B)** glucose utilization. The reference (control) medium was ZM1. The CSL medium contained either 4 or 10 mL of cCSL/L TW. In one case, ammonium chloride (1.6 g/L) was used as a N supplement. All media contained about 10% (w/v) glucose. The pH and temperature were kept constant at 5.0 and 30°C, respectively.

(Fig. 2). In all cases, the glucose-to-ethanol conversion efficiency was ≥98% theoretical maximum (results not shown). At a volumetric supplementation of 2%, the fermentation performance was similar to that achieved in the semisynthetic reference medium (Fig. 3). Addition of inorganic nitrogen (NH_4Cl) proved to be an effective means of decreasing the level cCSL required for growth and fermentation (Fig. 2). In a similar fashion, Fig. 3 shows the response by Z. *mobilis* CP4, in terms of cell mass and rate of glucose utilization, to the amount (mL) of cCSL added to a medium consisting solely of autoclaved TW and either 4 or 10% glucose. At the lower glucose concentration, the cell mass reaches a plateau of 1.6 g DCM/L (correspond-

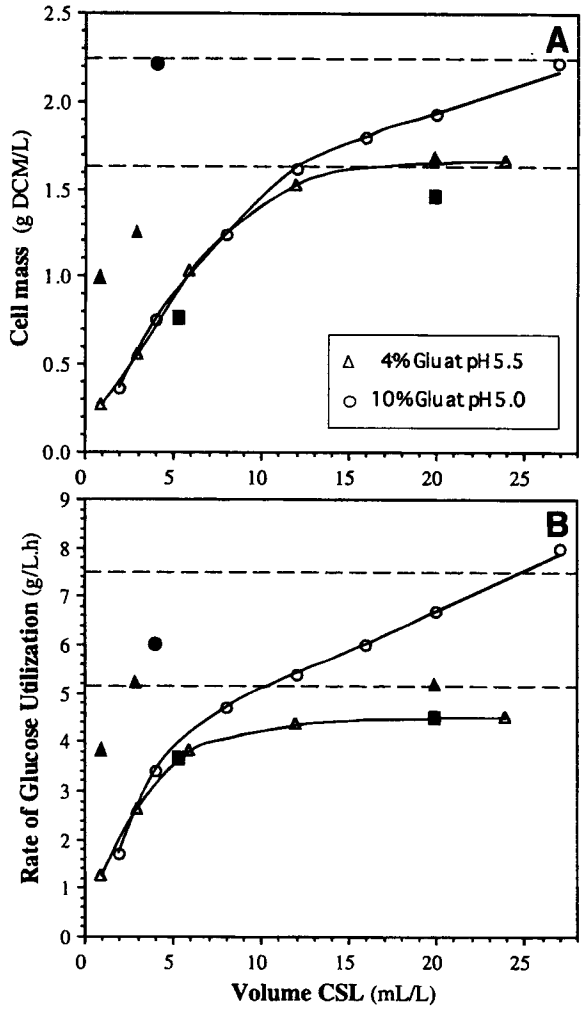

Fig. 3. Effectiveness of CSL in terms of volumetric rate of medium amendment. **(A)** Cell mass and **(B)** rate of glucose utilization. The media consisted TW with either 4% (w/v) or 10% (w/v) glucose with pH controlled at 5.5 and 5.0, respectively. The filled square symbols represent fermentations conducted with wCSL, and in all other cases, CSL was used. In some fermentations, the medium was supplemented with ammonium chloride: the filled triangles represent addition of 1.2 g/L to the 4% glucose-cCSL medium, and the filled circle represents the addition of 1.6 g/L to the 10% glucose-cCSL medium. The dashed lines represent the levels observed with the semisynthetic reference medium (ZM1) with either 4 or 10% glucose.

ing to the cell density achieved with the semisynthetic reference medium) at about 15 mL cCSL (1.5% v/v) (Fig. 3A). In the case of the rate of glucose utilization, the maximal rate is achieved at about half the amount of cCSL, but is less than the rate observed with the ZM1 medium (Fig. 3B). The solid square symbols in Fig. 3 represent the addition of 5.5 and 20 mL of nonclarified CSL (wCSL) to a TW (4% glucose) medium. Although the cell mass level using wCSL is lower than for comparable volumes of cCSL (Fig. 3A), the rate

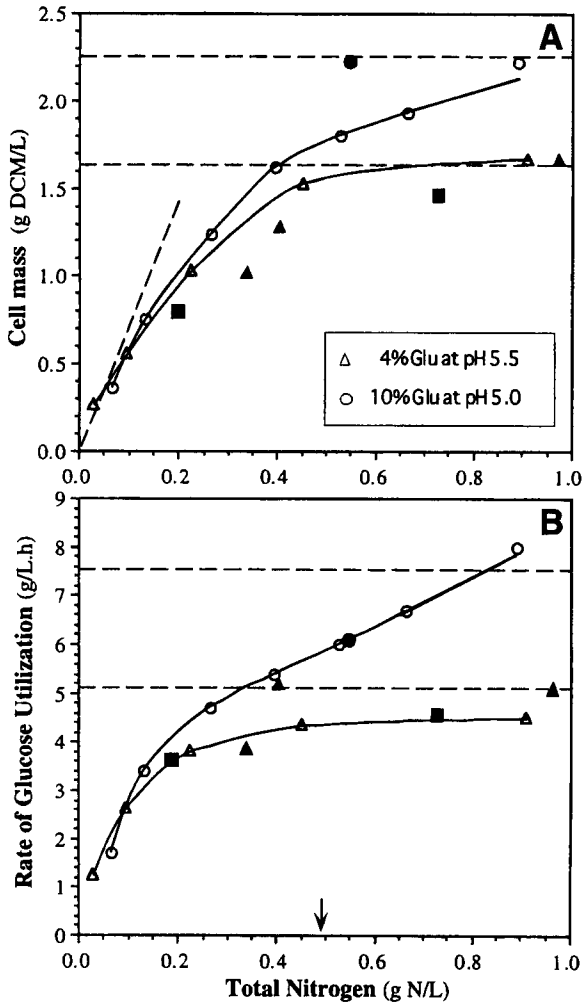

Fig. 4. Effectiveness of CSL as a function of the total nitrogen content of the medium. **(A)** Cell mass and **(B)** rate of glucose utilization. The conditions and symbols are the same as for Fig. 3. The *x*-axis arrow indicates the total N content of the reference medium (ZM1). The tangential dashed line in panel A shows the predicted response in terms of cell mass and is based on 100% assimilation of the N in the medium and *Zymomonas* dry mass assayed at 13.6% N.

response appears indifferent (Fig. 3B). From Fig. 3 it can be estimated that it would take about 1.3 times more CSL to achieve the same cell density as cCSL. With 10% glucose, the amount of cCSL required to achieve a cell density and fermentation rate comparable to the reference medium is increased to >25 mL/L with an apparent linear dependency for both responses in the range 12–25 mL cCSL/L (Fig. 3). In the Fig. 3, the filled triangles and circle represent experiments where the medium was fortified with NH_4Cl. For low-level cCSL supplementation, addition of inorganic nitrogen increases both the cell density and the rate of glucose utilization (Fig. 3).

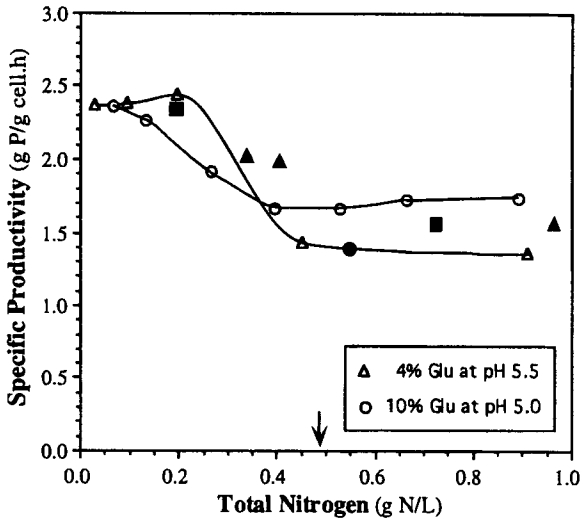

Fig. 5. Specific productivity as a function of the total N content of the medium. The conditions and symbols are the same as for Fig. 3. The *x*-axis arrow indicates the total N content of the reference medium (ZM1). The specific productivity was estimated as the ratio of the average rate of glucose utilization to the cell mass concentration, multiplied by the ethanol mass yield (which was relatively constant at 0.5 g/g).

The batch fermentations shown in Fig. 3 employed two different samples of CSL, and for the purpose of standardizing the data, the responses were plotted as a function of the N content of the medium (Fig. 4). The arrow on the abscissa indicates the N content of the reference medium ZM1 (Fig. 4B). In general, Fig. 4 shows that both the cell mass and rate of glucose utilization respond in accordance with the level of N in the medium up to a level of about 0.5 g N/L. The fact that maximal cell density is not achieved at this level of N with 10% glucose may relate more to a deficiency with respect to an essential growth element that is different from N—one possibility is pantothenic acid for which *Zymomonas* is known to have a specific growth requirement *(23)*. In all fermentations, the ethanol mass yield was ≥0.50 g ethanol/g glucose. Figure 5 shows the response of *Z. mobilis* CP4 in terms of specific productivity (g ethanol/g DCM/h) to the N content of the cCSL media. The increase in specific productivity at the growth-limiting levels of N is confirmation of previous observations with N-limited growth of other cultures of *Zymomonas (11,18)* where this condition leads to an energetic uncoupling of growth and glucose utilization.

Whereas for the purpose of defining minimal level of nutrients, it is imperative to minimize nutrient transfer for the seed culture medium at the time of inoculation, from a practical perspective, seed cultures are introduced to the batch fermenter at a volumetric "pitch" rate of about 5–10%. To determine the effect of nutrient transfer, we conducted a series of fermentations in which variously produced seed cultures were trans-

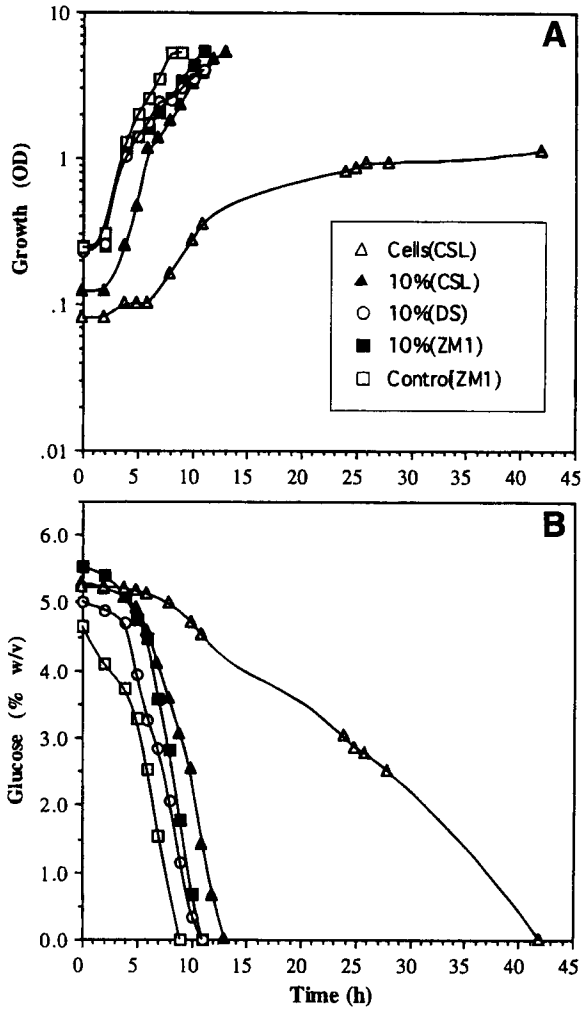

Fig. 6. Time-course of batch fermentations with *Z. mobilis* CP4. **(A)** Growth and **(B)** glucose utilization. The reference (control) medium was ZM1. The medium contained defined salts (DS), but was lacking the vitamins pantothenate and biotin (*see* Table 1). Three different media were used to generate the seed cultures for inoculation of the vitamin-deficient DS medium. For the purpose of seed production, the medium was (1) ZM1, (2) TW + 2% (v/v) cCSL, or (3) DS. A 10% (v/v) inoculum was used, except in one case where the seed culture grown in the CSL medium was harvested by centrifugation and the resuspended cells ("cells") were used as inoculum.

ferred into a minimal defined salts medium that did not contain the normal complement of vitamins. The following three media were used for the seed cultures:

1. TW with 20 mL/L cCSL;
2. A defined salts (DS) medium (Table 1); and
3. ZM1.

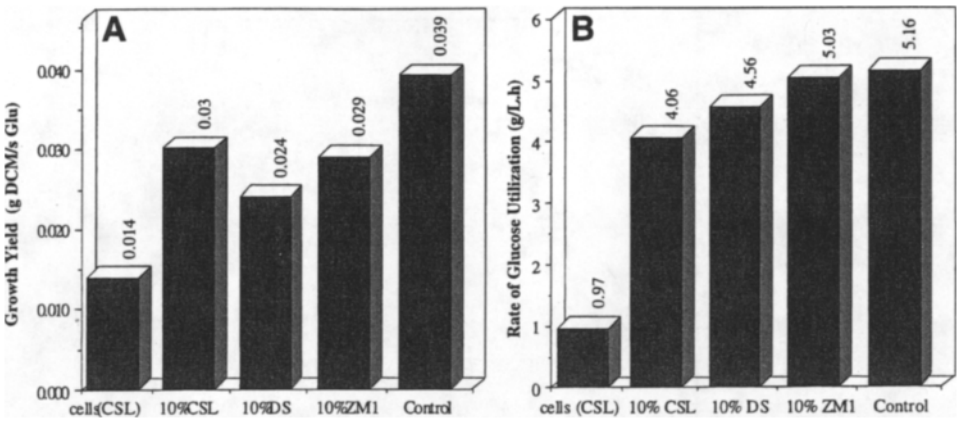

Fig. 7. Effect of nutrient transfer from seed culture on fermentation performance of *Zymomonas* in a defined salt medium lacking vitamins. **(A)** Growth yield and **(B)** rate of glucose utilization. The experimental design and conditions were as described in Fig. 6.

Figure 6 shows that surprisingly even the harvested cells could grow in the vitamin-deficient salts medium. The low level of growth may reflect a phenomenon known as "crossfeeding"—a form of microbial cannibalism. Where there was liquid culture used for inoculation, growth and fermentation were almost indistinguishable from the control using the relatively nutrient-rich ZM1 medium (Fig. 6). The responses in terms of cell mass concentration and glucose utilization rate are represented by the bar graphs in Fig. 7. These experiments clearly demonstrate that, at an inoculation ("pitch") rate of 10% (v/v), nutrient transfer is significant, and this practice could dramatically reduce the requirement for nutrient supplementation of the bulk fermentation medium.

Economic Impact of Using CSL as a Nutritional Adjunct in Large-Scale Fermentations

In terms of formulating a cost-effective fermentation medium that contains saturating yet minimal amounts of the essential elements for growth of the biocatalyst, cost reduction is a relatively facile exercise when the reference medium is comprised of research-grade lab chemicals. For example, the estimated cost of ZM1 medium, based solely on the use of industrial-grade yeast extract (US $3.30/lb), is US $21.80/1000 L. In a recent economic study based on a biomass-to-ethanol process using recombinant *E. coli*, it was suggested that inorganic chemicals could supply all the required elements for growth, and from the figures quoted in the report, the cost of the medium was determined to be 71¢/1000 L *(44)*. Although the efficacy of such a defined medium formulation was not tested, it still represents a cost of US $0.106/gal of denatured ethanol. According to current

economic considerations for improving process efficiency, such a high cost for nutrients is viewed as unacceptable *(20)*.

Low-level use of CSL as a sole nutritional supplement in large-scale fermentation operations represents an opportunity for apparent significant cost reduction with recent independent estimates being US $0.042/gal ethanol in a biomass-to-ethanol process using hydrolyzates that contained 6–8% (w/v) fermentable sugar and a conversion efficiency of 90% *(30,39)*. However, both these estimates were erroneously based on CSL costing US $50/dry ton and consequently represent an underestimated cost impact associated with the use of CSL as a sole nutrient supplement. In reality, the cost impact should have been about double that estimated previously, namely, US $0.084/gal of ethanol.

Based on the nitrogen content of *E. coli*, Grethlein and Dill *(39)* have estimated that it would require 7 g dry wt (DW) CSL/L to achieve a cell density of 3 g DCM/L. At about 40–45% (db) protein; the N content of 7 g dry CSL would be about 0.45 g N ($7 \times 0.4 \times 0.16$), and at an assumed N content for the cell mass of 15% (w/w), the cell mass would represent 0.45 g N. For CSL costing US $50/t, supplementation at a rate of 7 g DW/L amounts to a cost of 77¢/1000 L of fermentation medium. The literature contains several reports of the fermentation media formulations involving CSL, but in the majority of these investigations, economics was not considered, and there was no attempt to define minimal levels compatible with high fermentation performance. For example, Amartey and Jeffries *(35)* used 28 g/L CSL to replace YE and other nutrients in xylose fermentations by *Pichia stipitis*. In fermentations of corn crop residues by recombinant *E. coli* KO11, the medium was supplemented with 2% (v/v) wCSL (equivalent to 24 g total mass of CSL/L) *(37)*, or alternatively, at a rate 1–5% (v/v) in conjunction with a crude yeast autolyzate as an additional nutritional supplement, the exact amounts of each component was not specified *(40)*. In all of these studies, it was concluded that CSL was an effective substitute for expensive YE and protein hydrolyzate additives.

For CSL selling at US $50/t, medium containing 1% (v/v) cCSL would cost 88¢/1000 L. The cost of using nonclarified (wCSL) at the same supplementation rate of 1% (v/v) would be 66¢/1000 L. However, we observed that equivalent performance is achieved with proportionately higher levels of CSL, and consequently, the cost of using wCSL at 1.3% (v/v) is the same. However, 40% of the solids are insoluble and could potentially be sold back into the animal feed market for a byproduct credit, thereby reducing the cost of using 1% cCSL to 52.8¢/1000 L. If wCSL is used, the protein content of the insolubles will contribute both to the mass and to the feed value of the fermentation residuals.

The economic impact that is associated with the use of CSL as a sole nutritional supplement in terms of cost per gallon of ethanol produced depends on the sugar loading (Fig. 8). At a sugar loading of about 10% (w/v), the cost is 5.2¢/gal undenatured ethanol (Fig. 8). This cost is based on:

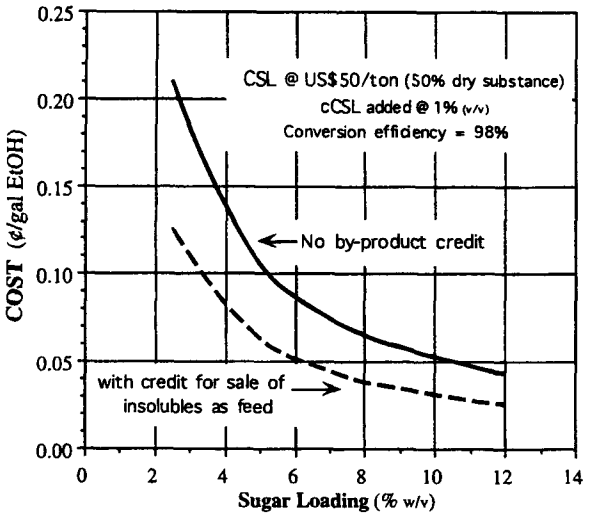

Fig. 8. Economic impact associated with use of CSL. Cost in terms of ¢/gal of undenatured ethanol was based on (1) using CSL as sole nutrient source, (2) CSL at US $50/t (50% solids), (3) supplementation rate using clarified CSL at 1% (v/v), (4) sugar-to-ethanol conversion efficiency of 98% by *Zymomonas*, (5) 100% product recovery, and (6) no by product credit for the sale of removed CSL solids (insolubles). The dashed line shows cost based on a by product credit for the sale of the CSL insolubles as feed at US $100/t.

1. CSL at US $50/t ("50% solids"—that is, 50% dry substance and therefore costing US $100/dry t);
2. The use of cCSL at a level of 1% (v/v);
3. Sugar-to-ethanol conversion efficiency by *Z. mobilis* of 98%; and
4. 100% product recovery.

An interesting aspect of this cost analysis is that although the cost of CSL is based on solids, 40% of these solids (i.e., the insolubles) are not used. Therefore, the cost impact is reduced by 40% (see dashed line in Fig. 8) if there is a byproduct credit for selling the insolubles as animal feed at a price of about US $100/t. Therefore, at a sugar loading of 10%, the cost of using 1% cCSL is reduced from 5.2 – 3.1¢/gal.

It can be concluded that low level CSL supplementation can supply the nutritional requirements compatible with growth and high-performance fermentation *of Zymomonas*, and that the disposition of the CSL insolubles can impact the calculations of cost associated with the use of CSL as a nutritional adjunct in large-scale fermentations.

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