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**Establish Medium Requirements for High Yield Ethanol Production
from Xylose by Existing Xylose-Fermenting Microorganisms**

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SUMMARY

Economic large scale production of ethanol from lignocellulosic materials requires efficient conversion of xylose to ethanol. The biocatalyst that was assessed in this investigation was the patented recombinant *Escherichia coli* B(ATCC 11303) carrying the *pet* operon with genes for both pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis* on a high copy plasmid designated as pLOI297 (US Pat. 5,000,000).

The purpose was to determine the minimal nutrient requirements for growth of the biocatalyst and to assess its fermentation performance characteristics using pure xylose. Another objective was to cost-effectively refine the selected defined medium to enable improved fermentation performance with respect to both ethanol yield and productivity. A complex and nutrient-rich medium (LB) was the reference medium that provided the benchmark with respect to performance standard in terms of both growth and xylose fermentation. This investigation involved several different media formulations ranging from complex with a variety of organic supplements to a chemically-lean defined minerals salts medium but it did not include biomass prehydrolysates. Hence, this study did not take into account the potential nutritional value of the biomass hydrolysate. Although pH 7 was shown to be sub-optimal for fermentation, hemicellulosic hydrolysates 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 medium was only about 15% compared to the reference medium. Amendment of the minimal medium with both vitamins and amino acids resulted in a performance that was still only 60% of that in LB.

Corn steep liquor (CSL) at about 7-10g/L was shown to be a complete source of nutritional requirements and supported a fermentation performance that approached that of LB. At a cost of CSL of \$50/ton, the economic impact of using CSL as the sole nutritional supplement in a cellulosic ethanol plant was estimated to be about 4¢ per gallon of ethanol.

Nutritional requirements were assessed within the context of a batch fermentation process and we did not examine the effect of multiple sequential batch fermentations whereby a portion of the stillage would be used for the purpose of seeding the next batch. 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. In order to minimise potential loss of the ethanogenicity of the plasmid-bearing biocatalyst, a selective pressure was maintained throughout this study by including antibiotics in all media. In large-scale operations, even if antibiotics are required only for the generation of inocula, we estimated that the cost of antibiotic(s) for the purpose of ensuring and maintaining high-performance of the biocatalyst, is as important a consideration as the cost effectiveness of nutrient supplementation since the cost surpasses that of the CSL supplementation.

INTRODUCTION

Background

Fuel ethanol from biomass and the economic impact of xylose conversion

Lignocellulose remains recalcitrant to bioconversion because the yeast cultures presently employed in starch-based fermentations are unable to utilise the five-carbon pentose sugars that comprise the hemicellulose (HC) component (range 10-35% dry wt). Techno-economic analyses have consistently identified the "pentose conversion problem" as the target with the highest economic impact on biomass-derived fuel ethanol. The hemicellulose component of hardwood is particularly rich in xylose. The theoretical max. yield of ethanol from 1 oven-dried tonne of hardwood is about 474L, with 150L (30%) being potentially derived from the hemicellulose (xylose). To make wood-derived ethanol competitive with corn-derived ethanol would require reducing production costs to \$0.34/L and economic analyses indicate that this target cost could be achieved solely through the efficient utilization of xylose - even at feedstock costs of \$42 per oven dried tonne (Hinman *et al.*, 1989).

Xylose utilizing ethanologens

Current processing technologies employing dilute sulphuric acid are capable of yielding a 'prehydrolysis' stream containing a maximum xylose concentration (without concentrating) of about 60 g/L. R&D in this area is directed to designing a biosystem capable of producing fuel ethanol from hemicellulose hydrolysate at a pentose conversion efficiency (>70%), thereby surpassing that of current bioconversion technologies that propose to use pentose-fermenting yeasts (Skoog and Hahn-Hägerdal, 1988; Prior *et al.*, 1989) or thermophilic bacteria, which generally suffer from a relatively low tolerance to ethanol (Lynd, 1989).

Ethanologenic recombinant Escherichia coli

Although *Escherichia coli* is capable of metabolising both hexoses and pentoses, the by-product of anaerobic metabolism is a mixture of organic acids with ethanol sometimes appearing as a minor end-product. Using recombinant DNA technology, *E. coli* has been genetically transformed with the so-called *pet* operon carrying both pyruvate decarboxylase and alcohol dehydrogenase II genes from *Z. mobilis* CP4 (Ingram and Conway, 1988) and one of several constructs (*E. coli* Luria strain B carrying the plasmid pLOI297) has been judged to possess superior 'hardiness' (Ohta *et al.*, 1990) and ethanologenic characteristics. This construct is reported to produce ethanol >4% (w/v) at near max. theoretical efficiency from D-xylose, in a nutrient-rich, synthetic medium (Alterthum and Ingram, 1989) and is the basis of US Patent 5,000,000 (Ingram *et al.*, 1988).

The physiological characteristics of this plasmid-bearing recombinant (*E. coli* B pLOI297) has been extensively investigated (Alterthum and Ingram, 1989; Ohta *et al.*, 1990; Beall *et al.*, 1991)

and is the subject of the present study. We received a copy of this recombinant *E. coli* culture from L. O. Ingram (University of Florida) in December of 1989 and have continued working with it since that time (see Lawford and Rousseau 1991-1995).

Experimental Rationale

Strategies for Determining Nutritional Requirements of the Biocatalyst

Strategies for investigating microbial nutritional requirements through culture media formulation is comprehensively reviewed by Guirard *et al.* in *Manual of Methods for General Bacteriology* (1981). Another excellent resource reference for this area of investigation is the *Manual of Industrial Microbiology & Biotechnology* (Demain and Solomon, 1981).

Complex media

The classical model for the determination of nutrient requirements of a new microbial isolate involves starting with a nutritionally complex medium that supports good growth. The majority of nutrients in a "complex" or "undefined" culture medium are provided by extracts or enzyme digests of plant or animal products. Yeast extracts are commonly used because they are rich in vitamins and growth factors. Digestion of proteins from a variety of sources (eg. meat, milk, plants) by proteolytic enzymes, such as trypsin (Tryptones) or pepsin (Peptones), yields mixtures of readily assimilable peptides and amino acids. Such organic nitrogen is often referred to as 'preformed nitrogen' since the organism is not obliged to synthesize amino acids *de novo*. These complex nutritional supplements are available commercially in various degrees of refinement (purity) ranging from the most expensive highly refined laboratory/research grade products to less expensive technical-grade products. The product specification sheets reflect this degree of 'purity' of these products in terms of inorganic elements and thereby provide very useful information in terms of formulating media that are less complex.

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 environment (aqueous medium) supply the elements of carbon, nitrogen, phosphorous and sulphur 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 and are therefore referred to as "trace elements". These trace elements are primarily the metals such as manganese, copper, cobalt, molybdenum, zinc, etc. 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, it is important to understand in this context that cellular composition can be influenced by the chemical nature of the growth environment. Table 1 provides examples of elemental composition both with specific reference to *E. coli* and bacteria in general.

Defined media

The ultimate goal in determining the nutritional requirements of an organism is the development of a chemically “defined” medium that is quantitatively comprised of known ingredients added to distilled water. The term “synthetic” medium is sometimes used as an alternative to “defined” medium (Gerhardt *et al.*, 1981). Progression from a complex medium to one of defined composition usually involves a series of so-called subtraction trials in which the nutritional components are tested for effect one at a time. The order for testing should follow that shown in Table 1 (ie. in order of decreasing contribution to the elemental biomass composition).

Following the carbon source (which is established as xylose in this investigation), the next most abundant nutrient is nitrogen. The complex source of nitrogen is first replaced by sources of organic nitrogen such as urea, glutamate or glutamine followed by replacement by inorganic ammonium salts or nitrates. Since yeast extract is a known source of vitamins as well as other undefined growth factors, it should be possible to replace it with a defined mixture of different vitamins. Subsequent testing for the essential nature of each component would occur through subtraction of one component at a time. Similar experimental design strategies are typically employed for testing the growth requirements of the various other minerals and “trace elements”.

Although defined media are typically much more expensive than complex media, nevertheless they are often used in the production of value added substances (eg. specialty chemicals, drugs, etc.) because they provide a means of standardisation as well as tending to ensure process reproducibility. When the nutritionally essential elements are present in non-excessive amounts such that “balanced” growth occurs (ie. growth is not restricted or limited by the concentration of any element), the defined medium is described as being “chemically lean” or “minimal”. The disadvantage of using a defined medium is the associated reduction in growth rate and productivity. Therefore, for reasons associated with both increased cost and reduced productivity, defined fermentation media are rarely used in the production of bulk chemicals.

Semi defined media

When a single source of a complex nutritional supplement (extract or digest) is added to a chemically defined medium, the resulting formulation is termed a “semi defined” (“semi-synthetic”) medium. As the name implies, semi defined media are transitional between defined and complex media and are typically the product of subtraction refinements of complex media aimed at cost-reduction. The addition of relatively small amounts of organic (complex) nutrients supply growth factors and vitamins that significantly increase growth compared to a fully defined medium.

***Escherichia coli* culture media**

Because *Escherichia coli* has been so popular for bacterial geneticists and molecular biologists, there exists a virtual plethora of different recipes for culture media. These formulations cover the entire range of media types from nutritionally rich complex to chemically lean defined media.

Several different examples of *E. coli* culture media are cited in the ATCC catalogue of bacterial cultures and in *The Handbook of Microbiological Media* (see ref. Atlas, 1993). The composition of a few representative examples are compared in Table 2 below .

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 (Hinman *et al.*, 1989). The efficient utilization of the hemicellulose component of lignocellulosic feedstocks offers an opportunity to significantly reduce 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 (Hinman *et al.*, 1989). 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, NREL has established target values for each of these fermentation process parameters (McMillan, 1994). The target for ethanol yield (based solely on xylose) is 0.46g/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 maximum ethanol concentration is 25 g/L and for volumetric productivity it is 0.52 g ethanol/L/h (McMillan, 1994).

Factors affecting the selected performance parameters

Yield

The condition of high ethanol selectivity demands that production of other metabolic end-products be minimised. The recombinant *E. coli* has been metabolically engineered such that under anaerobic conditions xylose catabolism is directed almost exclusively to the production of ethanol and carbon dioxide. However, certain organic acids are also produced in minor amounts. Specifically acetate, succinate, lactate and formate are known to be produced. The ethanol yield is routinely based on the amount of xylose added to the medium and it is important in the case of rich complex media to ascertain the potential contribution of fermentable substances other than xylose to ethanol production. In addition to control fermentations with rich media in the absence of added xylose, the routine calculation of the carbon balance gives an indication of the potential for yield inflation by nutrients other than xylose when closure is exceeded. Another contribution to yield reduction in fermentations employing recombinant *E. coli* is genetic instability. The *pet* operon (plasmid pLOI297) contains antibiotic resistance markers for both ampicillin and tetracycline. Therefore, to minimise instability (ie. the loss of *pet* expression), all media were prepared with antibiotics.

Ethanol concentration

The final ethanol concentration is a function of the amount of available fermentable carbon source and the ethanol tolerance of the biocatalyst. To achieve the target concentration of 2.5 % (w/v) would require 5.3 % (w/v) xylose based on an assumed xylose conversion efficiency of 90% theoretical maximum. Although *E. coli* is less ethanol tolerant than yeast (or *Zymomonas*), the target amount of 25 g/L is well below the inhibitory threshold for recombinant *E. coli* (Ohta *et al.*, 1990). An important factor affecting ethanol concentration is temperature. Although the optimum temperature for growth of *E. coli* is 37°C, higher ethanol concentrations are achieved at a lower temperature. For this reason, 30°C was selected as the standard operating temperature.

Productivity

Since the volumetric productivity is a function of the biocatalyst concentration (cell mass) as well as the metabolic activity of that cell mass specifically directed to ethanol production, both the rate of cell mass synthesis (growth) and the final cell concentration are important parameters to be considered when examining the effect of medium composition on performance. In the absence of inhibitory effects either by endogenous or exogenous substances, the final cell concentration is a function of the growth yield co-efficient and the concentration of the growth limiting nutrient. In addition to growth, the cell concentration and consequently also the productivity is affected by the “pitching rate” or inoculum (ie. the amount of cell mass added to initiate the fermentation). In comparative studies, the inoculum size is generally standardised; however, caution should be exercised in comparing the results of different investigators unless differences in inoculation density are properly taken into account. For this reason the specific productivity is the preferred reference parameter.

OBJECTIVES

- (1) to determine the minimal nutrient requirements for growth of recombinant *Escherichia coli* B (ATCC 11303 carrying the *pet* plasmid pLOI297) and to assess its fermentation performance characteristics using pure xylose.
- (2) to cost-effectively refine the selected defined medium to enable improved fermentation performance of the biocatalyst with respect to both yield and productivity

* in the absence of receiving biomass prehydrolysate from NREL, the original SOW was limited to the above two objectives

MATERIALS AND METHODS

Organism

The patented recombinant *Escherichia coli* B (ATCC 11303 carrying the *pet* plasmid pLOI297) (Ingram *et al.*, 1988; Alterthum and Ingram, 1989) was a gift from L. Ingram (University of Florida, Gainesville, FL, USA) and was received in December, 1989.

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 20ml/dl) and sodium citrate (1.5g/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 (Ingram *et al.*, 1987; Lawford and Rousseau, 1991).

Fermentation equipment

Batch fermentations were conducted in either 125ml shake flasks or 2L bench top stirred tank bioreactors (STR). Shake-flask fermentations were conducted in 100ml of buffered medium in 125ml screw-cap Erlenmeyer flasks in a water-bath shaker (New Brunswick Scientific Co., model G76) The agitation was set at about 100 RPM and the temperature was constant at 30°C. pH-stat STR batch fermentations were conducted in a volume of 1500ml in MultiGen™ (NBS model F2000) stirred-tank bioreactors fitted with agitation, pH, and temperature control (30°C). The central shaft was fitted with three multi-blade turbine impellers and the rotation speed was constant at about 100RPM. The pH was monitored using a sterilisable combination pH electrode (Ingold) and was controlled at 7.0 by the addition of 4N KOH (NBS model pH-40 controller). On average, about 75ml of 4N KOH was added during the fermentation of 4% xylose in STR pH-stat batch fermentations.

Methods of pre-culture and inoculation procedures

A 1ml 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 (NBS gyrotory shaker, model G76, at about 100 RPM). Alternatively, in the case of experiments employing defined media, the inoculum was prepared by transferring an aliquot of a glycerol/citrate preserved culture to a defined salts medium (Lawford and Rousseau, 1991) containing about 20g/L xylose and antibiotics (40 mg/L ampicillin and 10 mg/L tetracycline).

Flask fermentations were inoculated by transferring about 5-10ml of the overnight culture directly to 100ml medium in the flask. STR batch fermentations were inoculated by transferring approximately 100 ml of the overnight flask culture directly to 1400 ml of medium in bioreactor. For both flask and STR fermentations, the initial cell density was monitored spectrophotometrically to give an OD₅₅₀ in the range 0.1 - 0.2 (unless stated otherwise), corresponding to 30-50 mg dry wt. cells /L.

In the latter stages of this investigation, an alternative pre-culture and inoculation procedure was developed which permitted pre-culture using LB and which avoided typical centrifugal harvesting/washing procedure but which also minimised the potential for transfer of nutrients during inoculation. Pre-culture was accomplished using LBA⁺ (amp/tet) 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 defined minimal medium (MM8). The cell suspensions from several plates were pooled, agitated to achieve homogeneity and used to inoculate the STR at the desired initial cell density (OD). This procedure was designated as the "plate direct inoculation" (PDI) method.

Fermentation media

For comparative purposes, the nutrient-rich, complex culture medium described by Luria and Delbruck (1943) was used as the bench-mark standard. This medium is commonly referred to as "Luria broth" (LB) and consists of 5 g Difco Yeast Extract, 10 g Difco Tryptone and 5 g NaCl per litre of distilled water. Two different modifications of this LB medium were used in this investigation. Supplemented LB (sLB) was fortified with magnesium (0.5mM added as MgSO₄) and phosphate (17mM). Modified LB (mLB) contained reduced amounts of yeast extract (2.5 g/L) and tryptone (5 g/L) and no NaCl.

In the case of experiments with semi-defined and defined media, the basic defined salts medium of Lawford and Rousseau (1991) was amended by specific supplementation (vitamins and amino acids) as described. The basic defined salts medium (designated as "MM8") was comprised of 2.25g NH₄Cl; 0.1 g MgSO₄; 7mg FeCl₃.6H₂O; 2.72g KH₂PO₄; 3.48g K₂HPO₄; 5.0g NaCl; 12mg CaCl₂.2H₂O; 9.9mg MnCl₂.4H₂O; 0.05mg thiamine and 0.21g citric acid per litre of distilled water (see also Tables 15 and 16)

For flask fermentations, the initial xylose concentration was about 20g/L and, for STR fermentations, it was usually about 40g/L, unless stated otherwise. All media contained antibiotics (40mg/L ampicillin and 10mg/L tetracycline). All media were sterilised by autoclaving at 121°C for 30 minutes. The sugar supplements were autoclaved separately. Antibiotics were filter (0.45µ pore size) sterilised and added to the sterilised medium after cooling. Flask culture media were buffered by the addition of 2M stock phosphate solution (final phosphate concentration was 0.1M - the pH

was adjusted initially to 7.0) which was autoclaved after addition of the phosphate.

Analytical procedures

Growth was measured turbidometrically at 550nm (1cm lightpath) (Unicam spectrophotometer, model SP1800). In all cases the blank cuvette contained distilled water. Culture dry weight was measured by micro filtration followed by washing and drying the filter to constant weight under an infrared heat lamp. Compositional analyses of fermentation media and cell-free spent media were determined using an HPLC equipped with a RI monitor and computer-interfaced controller/integrator (Bio-Rad Labs, Richmond, CA). Separations were performed at 65°C using an HPX-87H column (300 x 7.8mm) (Bio-Rad Labs, Richmond, CA). The mobile phase was 0.005M sulphuric acid (flow rate = 0.6ml/min.) and the injection volume was 0.02 ml. Standards for D-xylose, succinic acid, DL-lithium lactate, potassium acetate and ethanol were prepared from research grade chemicals using a micro balance and diluting with distilled water using a volumetric flask. The xylose standard was 2 wt. %¹ and all others were about 1 wt. %. These standards were run on a routine basis (ie. for each batch of samples analysed). Isopropanol was used as an internal standard by using it to dilute samples. On a non-routine basis, retention times (Rt values) were also determined for the following substances: formic acid, glycerol, glutamic, citric and phosphoric acids.

Determination of growth and fermentation parameters

Linear growth rates (mg dry wt. cells/L/h) were determined from plots of optical density (OD) versus time using the relationship $1.0 \text{ OD}_{550} = 0.34 \text{ g dry wt cells/L}$ (Beall *et al.*, 1991; Ohta *et al.*, 1991; Lawford and Rousseau, 1991). The mass-based cell yield (obs. $Y_{x/s}$, where s = xylose) was calculated by dividing the maximum cell density (dry wt. cells/L) by the concentration of sugar consumed. In experiments with LB media, the cell yield was corrected for the contribution of the LB nutrients to the total cell mass (cor. $Y_{x/s}$). This value was experimentally determined to be 0.41 g dry wt. cells/L (see also Fig. 2) (Lawford and Rousseau, 1991).

The ethanol (product) yield ($Y_{p/s}$) was calculated as the mass of ethanol produced (final concentration) per mass of sugar added to the medium and was not corrected either for the dilution caused by the addition of alkali or for the contribution from fermentable components other than 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 ($\text{max } Q_s$) was estimated from the maximum slope in plots of sugar concentration versus elapsed fermentation time. The corresponding values of volumetric productivity (Q_p and $\text{max } Q_p$) were calculated by multiplying the values of Q_s and $\text{max } Q_s$ by $Y_{p/s}$, respectively.

¹ The term "wt. %" is meant to be equivalent to % (w/v) { i.e. 2 wt. % = 20 g/L } - see labeling of y-axes in Figures

Since the cell (dry) mass was determined only as an end-point (ie. 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 post-growth phase (max Q_s) could be divided by the maximum cell mass to yield an estimate of the specific rate of xylose utilization (q_s). Hence, in certain cases, the value for the specific productivity (q_p) was estimated by multiplying q_s by the overall ethanol yield ($Y_{p/s}$).

Carbon balancing and determination of %C recovery

Carbon balances were calculated as described previously (Lawford and Rousseau, 1992 and 1993). The amount of carbon (C) in the sugar added (as carbon and energy source) and end-products is presented as milliequivalents C (MW divided by number of carbons). For cell mass, meqC was determined as the dry wt. cells divided by 0.0246 (Lawford and Rousseau, 1993). Carbon dioxide was not determined directly but was estimated by assuming that 1 mole CO_2 was produced per mole of ethanol or acetic acid. No account was made for the possible conversion of formic acid to CO_2 and H_2 or the consumption of CO_2 in the production of succinic acid. For the purpose of carbon balancing, the elemental composition of the cell mass was considered to be constant ($CH_{1.8}O_{0.5}N_{0.2}$) with a molecular weight of 24.6 g/mol. At 48.7% carbon, this composition of *E. coli* cell mass compares favourably to ones used by others at 47.1% (Diaz-Ricci *et al.*, 1992; Luria, 1960) and 47.4% (Stouthamer, 1979). Apart from cell mass, the major end-products were ethanol and CO_2 . Succinic and acetic acid were minor metabolic end-products of xylose metabolism by recombinant *E. coli* (pLOI297) under these conditions.

RESULTS and DISCUSSION

The complex, nutrient-rich, "reference" medium

A complex, nutrient-rich, medium that is commonly used in laboratory research experiments with *E. coli* is one known as Luria broth or LB (Luria and Delbruck, 1943). This medium is composed of 10g Bacto Tryptone; 5g Bacto Yeast Extract and 5g NaCl per litre of distilled water. 'Bacto' is a brand name of Difco Laboratories Ltd. (Detroit, MI, USA) and represents their research grade laboratory microbiological media components. Bacto Tryptone is a pancreatic enzyme digest of casein that is labelled as being "particularly rich in tryptophan" (Difco Labs). The composition of both Difco Yeast Extract and Bacto Tryptone is given in Appendix A. It should be noted that several variations of this medium are cited in the literature, for example Luria-Bertani medium (Atlas, 1993) contains twice the amount of NaCl (10 g/L NaCl). In their pioneering work on recombinant ethanologenic *E. coli*, Neale and co-workers used a complex medium composed of 16 g/L tryptone, 10g/L yeast extract and 5g/L NaCl (Neale *et al.*, 1988). In making comparative

analyses from the literature, it is important to understand that the composition of LB is 'standardised' only when the exact same amount and source of the complex supplements are employed.

Because Luria broth (Luria and Delbruck, 1943) had been used, almost exclusively, in the development of various ethanologenic constructs (Ingram *et al.*, 1987; Ingram and Conway, 1988) including studies specifically directed to physiological testing of recombinant *E. coli* B (pLOI297) (Alterthum and Ingram, 1989; Ohta *et al.*, 1990; Beall *et al.*, 1991), LB was selected as the "reference" medium in this investigation of the nutrient requirements of this organism. The "reference medium" provides a "benchmark" of fermentation performance of the test organism, *Escherichia coli* B (pLOI297). To maximize genetic stability all media used in this investigation, including the reference medium, contained antibiotics (ampicillin and tetracycline). Figure 1 shows a typical time-course for a pH-stat STR batch fermentation with the recombinant culture in LB containing about 40g/L xylose.

From studies with ethanologenic recombinant *E. coli*, LB is known to be capable of supporting a maximum cell density of only about 3.5 g dry wt. cells/L (Ingram *et al.*, 1987; Lawford and Rousseau, 1991). Furthermore, the maximum cell density (dry mass) was found to be directly proportional to the amount of sugar consumed up to a maximum sugar concentration of about 3.3 wt % and the cell yield in LB without added sugar was 0.4 g DW cells/L (Lawford and Rousseau, 1991). Hence, from a nutritional perspective, growth in Luria broth is carbon (energy)-limited only when the concentration of the added sugar is less than about 33 g/L.

Magnesium is known to have a positive effect on the ethanol tolerance of ethanologenic yeast and bacteria (van Uden, 1989; Dombeck and Ingram, 1986; Osman and Ingram, 1987). Using LB containing 80 g/L xylose and buffered at pH 6.8 with 0.2M phosphate, neither magnesium (0.5 or 2mM) nor calcium (1 or 5mM) "improved growth or fermentation" of recombinant *E. coli* (pLOI297) (Ohta *et al.*, 1990). However, we have previously noted that supplementation of LB with 0.5mM magnesium (Ingram *et al.*, 1987) or with a combination of 0.5mM magnesium and 17mM phosphate (Lawford & Rousseau, 1992a; Lawford and Rousseau, 1995) is an effective means of stimulating growth and increasing the maximum cell density with LB containing greater than about 30-40 g/L fermentable sugar. Our designation for magnesium/phosphate amended LB is "sLB" (see *Materials & Methods*).

Establishing the fermentation "performance" standard

(experiments with the *reference* medium)

Growth characteristics

The general growth characteristics of the plasmid-bearing recombinant culture relative to its host (ATCC 11303) have been reported previously (Lawford & Rousseau, 1991 and 1992a). Figure 1 is typical of the growth of the recombinant in LB containing 40 g/L xylose at pH 7 (note that the y-axis is linear). Under anaerobic conditions, growth is predominantly linear with an initial short

exponential phase that produces about 0.4 gDW cells/L (Fig. 2) which, in terms of culture turbidity, is equivalent to an increase in OD of 1.1 units. Figure 2 is a plot of the final cell concentration as a function of xylose concentration. Figure 2 demonstrates that with LB a condition of carbon (energy) limitation only exists at xylose concentrations <35 g/L. The growth yield ($Y_{x/s}$) of the carbon-limited culture with respect to xylose is given by the slope of the line in Figure 2 and is 0.051g DW cells/g xylose.

Fermentation characteristics

A typical fermentation profile for xylose utilization and ethanol production is shown in Figure 1. The shape of the plot of ethanol as a function of elapsed fermentation time is partly determined by the fact that the ethanol concentration at the early stages of the fermentation falls below the detection limit of the HPLC detector (0.1g/L at normal sensitivity).

Whereas the host culture *E. coli* B (ATCC 11303) produces lactic acid as a principal product of anaerobic xylose metabolism (Lawford and Rousseau, 1991b), the recombinant produces ethanol as the major end-product. However, other acids appear as minor end-products and, of these other acids, succinic acid usually dominates. Our observations on end-product distribution confirm those made by Beall *et al.* (1991). Although formic acid was sometimes observed as a product in the earlier stages of batch fermentations, we never observed it in end-point analyses. It is likely that formate spontaneously decomposes to carbon dioxide and hydrogen. In general, it has been our experience that more acetic acid is produced at pH 7 than at pH 6.3. Lactic acid was normally produced in very minor amounts (Beall *et al.*, 1991). In the absence of antibiotics in the medium, we often observed higher levels of lactic acid and we used the appearance of this acid as an indicator of culture instability.

Effect of Inoculum Size on Volumetric Productivity

Since the size of the inoculum affects volumetric productivity, when this parameter is used in a comparative study it is important to be consistent with respect to the amount of cell mass added as inoculum. The effect of inoculum size on the fermentation performance of recombinant *E. coli* in xylose-containing LB has been previously reported (Beall *et al.*, 1991). Figure 3 summarises our observations with respect to the effect of inoculum size on productivity. Our standard procedure employs an inoculation density in the range 0.1 - 0.2 OD (on average, equivalent to about 50 mg DW cells/L). This inoculation density was selected on the basis of its practicability on an industrial scale of operation where pre-culture transfer for batch fermentations is usually at the level of 5-7 vol. %. Ingram's lab routinely inoculates at a cell density which is ten times greater than ours (Beall *et al.*, 1991).

In addition to the size of the inoculum the conditions under which it is prepared are also important. It has been observed with recombinant *E. coli* that "conditioning" by pre-culturing in xylose reduces 'lag time' (Neale *et al.*, 1988). Hence, unless specified otherwise, the inoculum was grown with xylose as the carbon source.

Establishing the Yield of Ethanol from LB in the Absence of Xylose

In this investigation, the ethanol yield was based solely on the amount of xylose added to the medium and because the reference medium contains fermentable carbon other than xylose, it was important to quantify the extent of ethanol production from the non sugar components of the medium. Batch fermentations were conducted anaerobically in capped 125 ml Erlenmeyer shake flasks containing about 100ml of buffered LB medium. The results are summarized in Table 4.

In this experiment 'mLB' represents "modified LB" where the Tryptone component was 2.5 g/L and the Yeast Extract component was also 2.5 g/L. In determining the amount of ethanol in the spent fermentations broths from these batch fermentations, the detector sensitivity of the HPLC was increased 4-fold over normal operating setting.

The amount of ethanol derived from the non-sugar components of the nutrient rich LB medium was 0.68 g/L. The amount of 0.51 g/L is considerably less than the amount of "approx. 0.2 wt.%" reported by others for the "modified" LB (see p428 in Grohmann *et al.*, 1995).

The value for $Y_{p/s}$ can be calculated as $8.31/16.4 = 0.507$ g/g; however, if correction is made for the amount of ethanol derived from the non-xylose components of the LB medium, the $Y_{p/s} = 8.31 - 0.68 / 16.4 = 0.465$ g/g. It should be noted that in flask fermentations with buffered medium, there is no dilution by alkali and hence there is no need to adjust the value of the ethanol yield.

Using the standard ethanol assay procedure with respect to the HPLC detector sensitivity, only 0.165 g/L ethanol was measured in a pH-stat STR using sLB medium without any added xylose (Expt. B140a and B140b). The lower limit for accurately determining the ethanol concentration is about 0.1 g/L under standard HPLC operating conditions (ie. at routine HPLC detector sensitivity).

Effect of pH

Acetic acid toxicity is an obstacle to the fermentation of biomass hydrolysates (Lawford & Rousseau, 1992), but since the inhibitory effect on recombinant *E. coli* is minimised at elevated pH (Lawford & Rousseau, 1993), a pH value of 7.0 was selected as the control set-point in pH-stat STR batch fermentations. The effect of pH on growth, xylose utilization and ethanol production is shown in Figure 4. In this experiment (Expt. B142) the amount of 4N KOH that was consumed during the fermentation was monitored. Table 5 presents a summary of the various fermentation parameters with respect to xylose utilization, ethanol yield and productivity.

Figure 5 shows the ethanol yield as a function of pH over the range 6 to 7. The observed $Y_{p/s}$ (ie. uncorrected value) is based on the maximum ethanol concentration (at the time of complete xylose utilization) and the xylose concentration as determined at zero time. The effect of correcting $Y_{p/s}$ for the contribution from non-xylose fermentables in LB is shown in Figure 6. Also illustrated in Figure 6 is the effect of correcting the $obs. Y_{p/s}$ for dilution by the added alkali titrant. Figure 7 shows the total amount of titrant added as a function of pH. The shape of the titration curve

(ie. titrant as a function of pH) is a function of the combined dissociation constants of the different organic acids (principally succinic and acetic) produced by the recombinant *E. coli*. and these acids produce a buffering effect around pH 6. Because the amount of alkali required to control the pH at a value near 7 is disproportionately more than for control at pH near 6, and since the pH control set-point under standard conditions was 7.0, we used 4N KOH in an attempt to minimise the titrant dilution effect which is clearly much more pronounced at pH 7 than pH 6. [Note: in experiments prior to B111, 2N KOH was used as titrant].

The corrected yield values ($cor. Y_{p/s}$) reflect the increased volume of alkali that is used at pH 7.0. At pH 7, a total of about 80ml of alkali (320mmol) was added to the fermentor (working volume of 1500ml) over the course of the fermentation. At pH 7, the observed ethanol yield is 0.436 g/g and the yield corrected for dilution by 4N alkali is 0.464 g/g (Fig. 5). At pH 7, the $obs. Y_{p/s}$ is estimated at 94% of the ethanol yield that has been corrected for dilution by the alkali titrant. Under the standard conditions of this study, the total amount of alkali added corresponds to about 7.6 mmol/g xylose consumed. Under similar conditions (except that the xylose concentration was 8%), Beall *et al.* (1991) reported consumption of only 2.5 mmol/g xylose consumed at pH 6.8 and 6.9 mmol/g xylose at pH 7.2; however, at 4% xylose, at pH 6.8, the amount of titrant was 3.8 mmol/g xylose. This observation suggests that, with LB medium, more alkali is required during growth than fermentation, since the cell concentration for both 4% and 8% fermentations can be expected to be very similar.

Figure 8 shows the relationship between ethanol yield (based on xylose added to LB medium) and the pH control set-point (data taken from Table 1 of Beall *et al.*, 1991). The physiological studies of Beall *et al.* (1991) used the same recombinant culture (*E. coli* B with plasmid 297) and LB medium with 8% xylose. It is interesting to compare the general pattern of the effect of pH on ethanol yield in Figures 5 and 8. Beall *et al.* (1991) noted that pH 6 was clearly sub-optimal with respect to yield (Fig. 8) and it is not understood why pH 6 was selected by Ingram's lab as the optimal pH for all subsequent work on various recombinants, both *Klebsiella* and *E. coli* constructs. In their fermentations at both pH 6.8 and 7.2, the observed and corrected yields were identical, 0.43 and 0.47 g/g, respectively (Fig. 8). Under their standard fermentations conditions (350ml LB medium and 2N KOH as titrant), the $obs. Y_{p/s}$ over the pH range 6.8 to 7.2 was 91.5% of the $Y_{p/s}$ corrected for dilution by alkali. In the experiments of Beall *et al.* (1991), the amount of alkali added at pH 6.8 and 7.2 was 100ml and 276ml, respectively (Fig. 9) and in the light of this information it is difficult to rationalise their reported corrected values for ethanol yield. Although these authors based the yield on added xylose, they do not discuss correction for the contribution from non-xylose fermentables.

Figure 10 shows the effect pH on specific productivity. The specific productivity was determined alternatively using values for the observed and dilution corrected ethanol yield (ie. q_s times either $obs. Y_{p/s}$ or $cor. Y_{p/s}$).

From these experiments it can be concluded that the optimum pH in terms of both yield and

productivity is closer to 6 than 7. This conclusion would appear to affirm Ingram's claim regarding the optimal pH value; however, their published data does not validate their claim regarding pH 6 as the preferred pH (see Fig. 8). A sub-optimal pH was selected in this study in anticipation of the potential benefit that would accrue in acetate containing prehydrolysate fermentations.

Standard conditions for flask and STR batch fermentations

The design nature of nutritional investigations whereby each element in the culture medium and the concentration thereof is assayed separately makes these studies equipment intensive. Only four pH-controlled STR bioreactors were available for this study and therefore, to accommodate the time line for this investigation, preliminary screening experiments were conducted using shake flasks and buffered media. To accommodate the buffering capacity of these media and to reduce fermentation time, the amount of xylose was only about 20 g/L. Although expedient, the disadvantages associated with this type of experimental design related both to the lack of pH control and the timing of the end-point. Furthermore, growth measurements (OD) were complicated by culture clumping that is known to occur in phosphate buffered media (Lawford and Rousseau, 1992a).

The conditions that were adopted for the routine operation of both shake flask and pH-stat STR batch fermentations are summarized in Table 6. Further details are given in *Materials & Methods*

Development of a cost-effective complex medium

The objective of this series of experiments was to test the effect of substituting less expensive substances for the different complex nutrient supplements in the reference medium on the fermentation performance of the recombinant test organism. The initial approach to cost reduction simply involved a reduction in the amount of yeast extract and tryptone. The amount of Yeast Extract (Difco) was reduced from 5.0 g/L to 2.5g/L and the amount of Bacto Tryptone (Difco) was reduced from 10 g/L to 2.5 g/L. Such a modified LB medium (designated in this study as "mLB") has been adopted by others (Grohmann *et al.*, 1994 and 1995) although Grohmann's formulation for a reduced strength LB medium did not include NaCl. It should be noted that this modified LB medium was incorrectly described as "quarter strength LB" (Grohmann *et al.*, 1994). Results from a separate study (Lawford and Rousseau, unpublished observations) indicated that the omission of NaCl from LB medium had no effect on the growth and fermentation performance of *E. coli*. Beall *et al.* (1991) examined the effect of NaCl in LB, but did not report on the effect of omitting NaCl from LB. It was reported that both the growth yield and ethanol yield were slightly higher at 100mM NaCl (5.9g/L) than 200mM (Beall *et al.*, 1991). The observations with LB regarding possible magnesium (phosphate) deficiency can be extrapolated to the use of mLB since the source of these elements rests entirely with the complex components (yeast extract and tryptone). Experiments to test this were conducted using glucose as carbon source but the results were equivocal and these experiments need to be repeated (data not included).

Another variation of the LB formulation involved adding 2.5 g/L Difco Yeast Extract and 5.0 g/L Bacto Tryptone (equivalent to half-strength LB) to a mineral salts medium (Fig. 11). The composition of the salts medium (designated as “MM8”) is described in *Materials & Methods*.

A subtraction refinement of this medium involving the elimination of the Bacto Tryptone component led to the formulation of a series of *semi defined* media initially involving decreasing amounts of Difco yeast extract (Fig 11). The effect of adding decreasing amounts of Difco Yeast Extract to the defined salts medium (MM8) on cell yield and the rate of xylose utilisation, is shown in Figure 12. By definition, in this study, the maxima for cell concentration and Qs is indicated by the reference medium which contains 10 g/L Tryptone in addition to 5 g/L Yeast extract (Fig. 12). Yeast extract is a complex mixture of essential nutrients and provides vitamins and other growth factors as well as amino acids and inorganic elements such as P and Mg (see Appendix A for compositional analysis). Hence, it is not surprising to observe that yeast extract, in combination with the mineral salts medium, is a sufficient medium for the recombinant *E. coli* and at a level of 2.5 g/L achieves 86% of the growth yield of LB. However, the high cost of this refined product prompted us to examine less expensive commercial sources of a less refined yeast extract as a potential alternative to Difco yeast extract. It was important that the source be able to supply large quantities commensurate with the requirements of large-scale fermentations. Table 7 lists the different commercial products that were tested. A series of flask fermentations (see Appendix C - expts. F012 to F015) were involved in screening for the effect of these substitutions on the rate of xylose utilization. For example, flask fermentation series F013 is shown in Figure 13. Using the reference medium (LB or sLB), with 20 g/L xylose, the fermentation was about 80% completed at 16h. The flasks were sampled at time zero and at 16h and 20h. The use of rich media with relatively low amounts of xylose provided a shorter fermentation time, but because these experiments were conducted overnight, there was often uncertainty with respect to the end-point and the interpretation of the results due to both the time interval between sampling and the lack of sufficient data. The use defined salts media in flask fermentations resulted in a more protracted fermentation which could also be conducted overnight, but which was more accommodating in terms of the frequency of sampling and therefore provided greater certainty with respect to end-point determination. pH-stat STR fermentations were conducted to examine the effect of substituting a variety of commercially available products for Difco yeast extract and tryptone. These STR batch fermentations are shown in Figures 14 and 15 and the results are summarized in Table 10.

Cost Reduced Media Formulations (development of *semi defined* media)¹

Since the anticipated maximum xylose concentration in biomass prehydrolysates is about 6 wt % (Hinman *et al.*, 1989), the maximum cell concentration could be expected to be about 3g DW cells/L (based on a presumed growth yield for xylose of 0.051g DW cells/g xylose - see Fig. 2). In this investigation the xylose concentration was only 4 wt % and the corresponding anticipated

¹ by definition, the use of tap water (TW) and/or more than 1 supplement makes these ‘complex’ media

maximum cell concentration would be accordingly two-thirds of this amount of cell mass (about 2 g DW cells/L). Based on an assumed cell composition ($\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$) the nitrogen content of the cells mass can be calculated as 11.4 wt.% N [% N in cell mass = $(0.2 \times 14) / (24.6) \times 100 = 11.4\%$]. Therefore, to satisfy the requirement for nitrogen, the medium must contain minimally 0.114g of assimilable nitrogen per gram of cell mass synthesised during growth. From specifications provided from the commercial suppliers (see Appendix A), the nitrogen content of various complex media ingredients can be determined and, from this information, the minimum amounts of these ingredients required to support a specified maximum cell concentration can be calculated (Table 8) as well as the nitrogen content of various complex media formulations (Table 9). The amounts of N shown in Table 9 are based, for the most part, on the total N content and do not therefore necessarily represent the amount of available or potentially assimilable N. Hence, these amounts are 'minimal' in more than one sense.

Luria broth is nutritionally "rich" and clearly contains a surfeit of nitrogen (Tables 8 and 9). The values in these Tables provide important information in terms of formulating a leaner medium and Table 9 illustrates that all media (including the defined salts medium with ammonium chloride as sole N source) contain sufficient N to support a cell density >3 g DW cells/L. However, in pH-stat STR batch fermentations with approx. 4 wt % xylose, none of the leaner media supported as high a maximum cell density as did either LB or sLB (Fig. 11 and Table 10).

Under the assay conditions of this study, significant cost reduction was achieved without drastically affecting the fermentation performance of the biocatalyst in terms of yield. While volumetric productivity is less in these media compared to the reference medium, this parameter is less reliable for comparative purposes since it can be easily modified by an increase in the inoculation density. In this study, we used a relatively low inoculation density in order to minimise nutrient transfer and "cross feeding". Cost reduction is a relatively facile exercise when the reference medium is comprised of research-grade lab chemicals. Based on the total cost of all ingredients (excluding xylose), we estimated the cost of the reference medium (LB) and the defined salts medium to be \$1.95 and \$0.28 per L. The results of this study show that it is possible to achieve a 95% reduction in cost through the substitution of highly purified lab-grade chemicals, Difco Yeast Extract and Tryptone, with less expensive alternative commercial sources of yeast autolysate (Veeprex B430) and casein pancreatic digest (Pancase S). One formulation was selected as the 'best' in terms of cost and fermentation performance (designated as "TW+VYE+ AP+P"). This medium does not use inorganic supplements but uses tap water (as a source of inorganic elements), ammonium phosphate (1.4 g/L), yeast extract (2.5 g/L) and peptone (1.0 g/L). The price quoted for large orders of Veeprex B430 and Pancase S (Champlain Industries Ltd., Quebec, Canada) was \$10.85/Kg and \$36.63/Kg, respectively. It was assumed that ammonium phosphate could be supplied by commercial fertiliser. Based on these cost figures, we estimated the cost of this medium to be about \$ 0.10/L (Table 11). However, in terms of the economic impact on large-scale fermentations, even a cost for nutrient supplements of 10¢/L, is clearly prohibitive since the impact on the cost of ethanol would be about \$10/gal.

Development of a Cost Effective Medium for Large-Scale Fermentations

Corn steep liquor (CSL) is a by-product of corn wet-milling and contains a rich complement of important nutrients to support robust microbial growth and fermentation. 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 chemical composition is described in Table 12. The process (light) steep water (LSW) is concentrated by evaporation to about 40-50% solids to produce heavy steep water (HSW) or corn steep liquor (CSL). The protein content is estimated from the determination of the total Kjeldahl nitrogen and hence this does not truly represent the amino nitrogen content (Tables 12 and 13).

In considering CSL as a nutrient source and in formulating media using CSL, it is important to recognize that the exact composition of CSL can be expected to be somewhat source and process dependent. In this investigation the source of the CSL was NACAN Products Limited (Collingwood, Ontario, Canada). The specifications provided by the producer indicated a solids content of 40-45% and a protein content (based on total N x 6.25) of 40-43%. The current selling price of this product is C\$ 60-80 per ton (pers. commun. Mr. Brian Sawyer, NACAN Products Ltd).

Our experiments with CSL were designed to examine the potential of this 'industrial waste' (sold primarily as animal feed supplement) as a complete complex medium and to compare the fermentation performance of the recombinant *E. coli* in the CSL medium relative to the standard reference medium. For this purpose the CSL was diluted with tap water and xylose was added at about 40 g/L. The tap water was used both to reduce cost and as a potential source of trace elements (metals). The chemical composition of Toronto tap water is given in Appendix B. The quantities of CSL used (per L of tap water) are shown in Table 13 and the values for N are based on 0.028g N/ml CSL (or 0.067g N/g CSL) and a sample at 43% solids.

Assuming (i) that the *E. coli* is 11.4% nitrogen (dry wt basis), (ii) that CSL is the sole nitrogen source for growth, (iii) that all the N is assimilable, and (iv) that the N utilization is 100%, it would require at least approx. 4 ml CSL/L (1.7g CSL/L) to provide sufficient N for a maximum cell concentration of 1g DW cells/L.

The results of these batch fermentations with CSL are illustrated in Figures 16 and 17 and the comparative process parameters are summarized in Table 14. In flask fermentations (F018), a medium containing 18ml CSL (about 8 g/L) in tap water was compared to LB (Fig. 16). This CSL-based medium was also fortified with either vitamins or vitamins plus 1g/L Difco Tryptone (Fig. 16). Although equivocal in nature due to paucity of sampling and uncertainty of precise endpoint, the flask fermentations (F18 #6-9) seem to suggest that supplementation by vitamins and/or tryptone (1g/L) only promotes better growth yield (increased cell mass), but has little effect on productivity. At the level of 2% xylose and without pH control, the CSL-based media appeared to produce a fermentation performance by the biocatalyst that approximated that of the reference medium.

In subsequent pH-stat STR batch fermentations (Expts. B121 and B124), the amount of CSL added to tap water was varied over the range 4.5 - 55ml. CSL proved to be an effective substitute for all the combinations of other supplements tested. Based on volumetric productivity ($\max Q_s$), about 90% equivalence with the reference medium (sLB) was achieved at the levels of CSL >25ml CSL (9.5g CSL) (Fig. 18). At the lower levels of CSL used, the rate of xylose consumption (ethanol production) appears to be disproportionately high in comparison to growth. Figure 19 shows the relationship between specific productivity (based on end-point values - see *Materials & Methods* for method of calculation) and the amount of CSL added. Clearly, the specific activity of the biocatalyst is enhanced at the lower levels of CSL. Figure 19 illustrates that the specific productivity (q_p) exhibited at the higher levels of CSL is equivalent to that exhibited by the recombinant culture in the reference medium.

Based on the nitrogen content of *E. coli*, others have estimated that it would require 7g CSL/L to achieve a cell density of 3g DW cells/L (Grethlein and Dill, 1993). Amartey & Jeffries (1994) used CSL (obtained from ADM at 45% solids) to replace yeast extract and other nutrients in xylose fermentations by *Pichia stipitis*. These investigators found that with yeast 28 g/L CSL gave equivalent performance to 10 g/L Difco Yeast Extract (pers. commun.- T. Jeffries' laboratory).

In a recent report to USDA on cost analyses associated with selected processes for the production of cellulosic ethanol, Grethlein and Dill (1993) make specific reference to the use of recombinant *E. coli* KO11 and CSL as a nutritional supplement "*Dr. Ingram has found that CSL at 20 g/L is equivalent to the more expensive nutrients.*" (page 8-2). The only reference to the use of CSL by Ingram that was found in the scientific literature (Beall *et al.*, 1992) involved supplementation of a corn hulls acid hydrolysate with 20ml CSL per L (equiv. to 9 g/L at 45% solids) and this medium was sterilised by ultrafiltration. The cost analysis by Grethlein and Dill (1993) was based on supplementation of the recombinant *E. coli* fermentation with CSL at only 7 g/L as per information supplied by Dr. Ingram (ref. pg 8-3, assumption #2 - "*CSL will be the only other nutrient required and 7 g CSL per litre of broth will be adequate*"). It was estimated that at a CSL selling price of \$50/ton, use of CSL at a level of 7 g/L (plant capacity of 25 million gal/y) would contribute \$0.042/gal ethanol produced².

In our experiments CSL was the sole source of nutrients (excluding the added xylose and the inorganic elements supplied by the tap water) and supplementation at the level of 7g/L is approximately equivalent to 18ml CSL/L (B121c and B124a). It should be noted in comparing our results to those of Beall *et al.* (1992) that all our media were sterilised by autoclaving whereas theirs were filter sterilised. 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

² Further discussion relating to the economic impact of using CSL as a nutritional supplement appears at the end of this report

amount of CSL in supporting good growth and xylose fermentation by recombinant *E. coli*. For CSL costing \$50/ton, supplementation at a rate of 7 g/L amounts to a cost of \$0.385/m³ of fermentation medium.²

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 SO₂ to the steep water which can yield substances in the CSL that are potentially inhibitory to bacterial growth. Fortunately, most of the sulphur 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 (LAB) 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.³ Propagation of LAB can be expected to also decrease the potential nutritional value of the CSL. It is not known how lactic acid (or other by-products of LAB metabolism) might affect the fermentation performance of the biocatalyst.

Development of a Defined Medium

Bacteria are known to grow on simple salts media that do not contain growth factors or other complex ingredients and the literature provides several different recipes for defined media for culturing *Escherichia coli*. Table 15 shows some selected examples of *E. coli* defined media that range from complex formulations involving vitamin and amino acid supplements to chemically lean formulations that use inorganic salts to provide the essential elements for growth.

In previous investigations with this same recombinant *E. coli* strain (Lawford and Rousseau, 1991; 1992a), we used a minimal defined salts medium adapted from *E. coli* Medium M63 (Table 15) that we designated as "MM6". Based on further research conducted during the fall of 1990, our original formulation was modified (designated as "MM8") primarily through supplementation with calcium and manganese, replacing ferrous sulphate by ferric chloride, and substituting anhydrous magnesium sulphate for the magnesium sulphate heptahydrate (Table 15). It is the oxidised form of iron (Fe³⁺) that is accumulated by bacteria (Ratledge, 1971). In these defined salts media (MM6 and MM8), citric acid was added as a chelator to increase the bioavailability of trace metal elements. According to Pirt (1975), "*Many minimal media recommended in the literature are defective because they do not contain a chelating agent which prevents precipitation of iron and other trace metals*" (p 135).

³ Note: The CSL used in expt. B141d (6% xylose) had been stored in the refrigerator for several months and contained a significantly elevated concentration of lactic acid (70g/L). Note also that the data shown in Tables 14 and 23 and in Appendix D (eg. expt. B141d) refer specifically to the amount of lactic acid produced by the recombinant biocatalyst during xylose fermentation and not to the total concentration of lactic acid

In chemically defined media, the formation of insoluble ion complexes can potentially compromise the bioavailability of certain essential elements. Calcium sulphate and calcium phosphate are only sparingly soluble. Because of the known potential for the formation of insoluble complex ions (MgNH_4PO_4 , MgKPO_4 and MgNaPO_4) during autoclaving, it is generally recommended that heat sterilisation of chemically defined salts media be accomplished in such a manner that magnesium and phosphate are kept separate (Pirt, 1975). The potential for precipitation of magnesium phosphate complexes is decreased when the pH is <7 during autoclaving. The defined salts media used in all our STR batch fermentations were autoclaved as a complete formulation and the pH prior to autoclaving was in the range 6.3 to 6.6. We did not observe post autoclaving precipitation of salts even after prolonged storage at room temperature in sealed vessels. However, we observed that salts precipitation was a potential problem at elevated phosphate concentrations such as those employed in buffered media in shake flask fermentations.

The biocatalyst being tested in this study is known to express high levels of *Zymomonas* pyruvate carboxylase (*pdc*) (Alterthum and Ingram, 1989) and this enzyme is also known to contain magnesium and thiamine pyrophosphate (Bringer-Meyer and Sahm, 1986; Neale *et al.*, 1987). The *Zymomonas* alcohol dehydrogenase (*adh* II) that was used to construct the *pet* operon (Conway *et al.*, 1987; Ingram *et al.*, 1987) is unusual in that it requires iron for activity (Scopes, 1983). This fact underscores the potential importance of these substances in the development of appropriate defined media for recombinants in which there is a high *pdc* and *adh* II content. For this reason, our defined salts formulations were supplemented with thiamine 50 $\mu\text{g/L}$ (Table 15). The thiamine content of the complex reference medium (1mg thiamine per L of Luria broth) (Table 15) was estimated from its concentration in both the Difco yeast extract and tryptone (Appendix A) and is the same as the amount of thiamine in *E. coli* R Medium (Table 15).

The term "balanced growth" refers to a condition in which growth is not limited by the availability of any nutrient other than the carbon (energy) source. Cell composition can provide clues regarding nutritional requirements and, apart from the major elements such as nitrogen and phosphorous, the compositional analysis with respect to the trace elements can be informative in the design of a defined culture medium. A chemically lean (minimal) defined medium should support balanced growth and often mimics the elemental composition of the cell mass. Since the composition of all microorganisms is very similar in terms of the major elements such as C, N, K, P, S and Mg, the formulations of most defined media are also very similar. In this study, the elemental composition of the recombinant *E. coli* was not analysed, but rather we have relied on analyses reported by others (see Table 1). For the purpose of this study, we relied on the same formula for cell composition that we used in previous publications (Lawford and Rousseau, 1993a and 1995), namely, $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$, which represents a C:N mass ratio of 4.3:1 (Diaz-Ricci *et al.*, 1992; Stouthamer, 1979). Accordingly, the carbon and nitrogen content were assumed to be 48.8 wt. % and 11.4 wt. %, respectively. This value for the nitrogen content of the cell mass was used to determine the minimal requirement for assimilable N in the medium (0.114gN/g DW cells). Since this compositional formula of bacterial biomass does not include any other elements, we have used

literature values in establishing values for minimal requirements for P and Mg. The phosphorus content of bacterial cells in general is about 1.5% of the dry mass; however, the content increases with growth rate and varies inversely with temperature (Pirt, 1975). This is equivalent to 15mg P per g dry cell mass or 0.484mM P for 1g DW cells/L

In Gram-negative bacteria, the molecular ratio of Mg:K:PO₄ is 1:4:8 (Pirt, 1975). This is equivalent to a mass ratio of P:Mg of 10:1 The growth yield for magnesium varies in the range 300 to 900 g dry cell mass/g magnesium which is equivalent to a magnesium requirement of 1.1 - 3.3 mg Mg per g dry cell mass or 45 - 135µM Mg for 1g dry wt cells/L.

Table 16 compares the elemental composition of various *E. coli* defined media. For comparative purposes, Table 16 also includes the complex "reference" media (LB and sLB) and a medium consisting solely of tap water (see Appendix B for elemental analysis of Toronto water) supplemented with 1.4 g/L of ammonium monohydrogenphosphate ("TW + AP").

Using nutritionally rich media (at pH 6.3), the growth yield co-efficient with respect to xylose ($Y_{x/s}$) has been determined to be 0.051 g DW cells/g xylose (Lawford and Rousseau, 1995). This value may be slightly less at pH 7 (Fig. 2). Therefore, under the standard assay conditions of this study (ie. ca 4 wt. % xylose), the maximal cell concentration can be predicted to be about 2 g DW cells/L (ie. 40 g/L x 0.051g/g = ca 2 g/L).

Table 17 compares the composition of defined media with respect to the three major elements N, P and Mg and gives the estimated minimal requirements based on literature values of average elemental composition of bacterial biomass. Synthesis of about 2 g DW cells is estimated to require 0.23g N, 0.03g P and 0.003g Mg. Table 17 shows that the defined salts medium used in this study satisfies this minimal requirement, Furthermore, the medium consisting solely of tap water and 1.4 g/L ammonium phosphate ("TW + AP") also satisfies this minimal requirement for these major elements (Table 17).

In a recently published technical and economical analysis of the production of fuel ethanol from lignocellulosic biomass (willow) using recombinant *E. coli* KO11, von Sivers *et al.* (1994) suggested that in large-scale fermentations the cell mass would increase from 1 to 3 g DW/L during each fermentation and that the nutritional requirements would be satisfied by a medium containing 0.4g N, 0.2g P and 0.02g Mg/L. They did not recommend a specific recipe or source for these elements. These estimates of von Sivers *et al.* (1994) are based on measurements of the stoichiometry these elements in cell mass and therefore are similar to the ratios shown in Table 17.

Nutritional investigations using batch cultures and minimal defined media can be complicated by the transfer of nutrients from the pre-culture medium during inoculation especially in the case where this procedure involves direct volumetric transfer (usually at about 5-10 vol. %). This is particularly problematic when a rich complex medium is used for pre-culture. We observed that

when LB was used to prepare the inoculum, nutrient transfer during inoculation into a minimal defined medium (MM8) resulted in an enhanced growth rate and final cell density (Fig. 20). Pre-culturing in minimal defined salts medium eliminates the possibility of transfer of complex nutrients and growth factors (Fig. 20). Attempts to minimise nutrient transfer from rich pre-culture media through harvesting by centrifugation and resuspending in the defined salts medium were unsuccessful because this procedure of inoculum preparation appeared to have pronounced detrimental effects of culture vitality as reflected by the poor growth and xylose utilization rates (Fig. 20). We found this observation particularly interesting in light of the apparent success of others (Beall *et al.*, 1992). In connection with our participation in the IEA xylose fermentation round robin, we observed that saline washing of LB grown cells that had been harvested by centrifugation produced unsatisfactory fermentation performance (see Hahn-Hägerdal *et al.*, 1994).

Whereas our defined salts medium (MM8) supports growth of the recombinant culture, both growth and ethanol productivity are only about 15% of rates achieved in the reference (LB) medium (Fig. 20 and Table 20). Growth in the defined salts medium did not appear to negatively affect end-product selectivity and although there is not complete closure with respect to the carbon balance (about 90% carbon recovery), the use of this medium provides the potential to determine the ethanol yield from xylose independent of contribution from other carbon sources. Clearly, the fermentation performance in this lean defined medium is far too poor to be of practical utility.

Effect of vitamin supplementation of the basic mineral salts medium

In the reference medium, yeast extract is used to provide vitamins and other growth factors.

“Vitamin requirements for microbial cultures have rarely been defined quantitatively in terms of the growth yield so that the amount required to produce a given amount of biomass is unknown.” (J. S. Pirt, 1975, p121).

For the purposes of this study, we formulated a vitamin ‘cocktail’ consisting of 8 vitamins including thiamine the composition of which is shown in Table 18. This formulation was based in part on our observations with CSL (see Table 18). We had established the sufficiency of 18ml CSL/L (about 7g CSL/L) to support good fermentation performance of the recombinant and our complex vitamin supplement reflects the stoichiometric amounts of these vitamins in CSL (Table 18). Our vitamin supplement formulation is not dissimilar to that of Hernandez and Johnson (1967) (Table 15) which was based on the vitamin content of bacteria (Luria, 1960).

We used shake flask fermentations to screen for the effect of supplementing the basic defined salts medium (MM8) with vitamins. Figure 21 (Expts. F018 and F019) suggests an enhancing effect of vitamin supplementation on growth and xylose utilization, but indicates that performance equivalence to LB is not achieved by such an amendment of the defined salts medium. In later experiments we decreased the amount of vitamin supplementation by 50% without apparent appreciable loss of this enhancing effect (see Expt. F025 in Fig. 23). However, when thiamine (10µg/L) and nicotinic acid (0.75 mg/L) alone were used to supplement MM8, the stimulating effect was noticeably less than with the complete vitamin mixture (Expt. F025 in Fig. 23).

Supplementation of the basic mineral salts medium with glutamate and/ mixtures of amino acids

In addition to vitamins (and other growth factors), nutritionally rich synthetic media often contain a relatively complete complement of the amino acids. The supply of amino acids in the culture medium by-passes the necessity for biosynthesis from inorganic nitrogen and carbon metabolites derived from xylose catabolism which results in a markedly enhanced rate of growth. The amounts of amino acids and vitamins in the complete defined medium used by Hernandez and Johnson (1967) to grow *E. coli* K12 (see 'H&J Medium' in Table 15) were based on the composition of bacteria (Stokes and Gunnes, 1946; Luria, 1960). Similarly, the complete defined medium used by Lee and Chang (1993) for the high cell density (>100g DW cells/L) cultivation of recombinant *E. coli* W (see 'R Medium' in Table 15) contained 16 amino acids. Table 19 shows the composition of these media with respect to the amounts of the different amino acids. Urea, glutamate or glutamine are commonly used as a readily assimilable form of organic nitrogen in defined media in combination with ammonium salts as a supplementary source of inorganic nitrogen (Demain and Solomon, 1981).

We used shake flask fermentations to screen for the effects of different levels of organic nitrogen supplementation to the vitamin enriched defined salts medium (ie. "DS + V"). Figures 22 and 23 show the effect of adding various amounts of glutamic acid (G). It would appear that too much glutamic acid (3 g/L) in the medium can be inhibitory (Fig. 22). While the results of our flask experiments did not provide conclusive information regarding an optimal level of glutamic acid supplementation, an amount of 0.25 g/L appeared to produce the best effect in terms of both growth, xylose utilization and ethanol productivity. This level of glutamic acid supplementation was tested in pH-stat STR batch fermentation (Expt. B134c) and the results are shown graphically in Figure 25 with the operational parameters summarized in Table 20.

The results are compared to the effect of substituting 2.5g/L tryptone for the glutamic acid (Expt. B133b; see Fig 25 and Table 20). Additional supplementation with aspartic acid, histidine, phenylalanine, tryptophan and tyrosine ("AA") produced no significant enhancement of growth and productivity (Expt. F026 - Fig. 24; see also B135c in Fig. 25 and Table 20). However, supplementation with a more complete amino acid cocktail ("AA⁺") consisting of 16 amino acids (50mg/L) in addition to glutamic acid (250mg/L), had a pronounced performance enhancing effect (Expt. B135d - see Fig. 25 and Table 20). Fermentation performance in this medium clearly does not rival that in the nutritionally rich and complex reference medium, but it comes close to the performance produced by the defined salts medium that had been amended with a vitamin supplement and 2.5 g/L tryptone (B133b). The specific rate of xylose utilization in the amino acid (AA⁺) supplemented medium is 90% of that in the medium amended with tryptone at 1g/L

Effect of trace metals supplementation of the basic mineral salts medium

According to Pirt (1975), “*In batch cultures, trace element deficiency is more likely to be reflected in a decrease in growth rate than growth yield*”. In an investigation of whey fermentation by recombinant *E. coli* KO11, Guimaraes *et al.* (1992) reported a beneficial effect on fermentation performance through supplementation with a mixture of trace metals. Supplementing whey with trace metals and ammonium sulphate reduced fermentation time and increased ethanol yield (Guimaraes *et al.*, 1992). The composition of the salts supplement and trace metals mixture used by Guimaraes *et al.* (1992) is shown in Table 21. The composition of the trace metal mixture was based on the formulation of Zabriski *et al.* (1988).

Guimaraes *et al.* (1992) reported that “*no decrease in fermentation rates or yields was observed after eliminating copper, cobalt and calcium from this trace metal mixture*”. In flask fermentation series F026 we tested the effect of using this same trace metals mixture (“TE”) and concluded that it had no beneficial effect on fermentation performance in the defined salts medium that was amended with both vitamins (V) and 0.25 g/L glutamic acid (G) (see Fig. 24).

Fermentations with 6% Xylose

The economic analysis of Hinman *et al.* (1989) 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.*” Using the values of NREL’s targets for operational parameters with respect to xylose conversion, processing of 6% xylose at a product mass yield of 0.46 (based on xylose) would yield an ethanol concentration of 27.6 g/L which is slightly higher than the targeted final ethanol concentration of 25 g/L (Table 3). Under the standard assay conditions used in this investigation the target ethanol concentration of 25 g/L could not be achieved even at a biocatalyst conversion efficiency of 100% since the xylose concentration was only about 4%. Therefore, in the final stages of our investigation we decided to examine the fermentation performance of the recombinant in selected media formulations at the upper limit of xylose concentration to be anticipated in dilute acid prehydrolysates (ie. ca 6 wt. % xylose).

Table 22 compares the composition of the selected media with respect to N, P and Mg. Based on the growth yield with respect to xylose, the maximum cell density that could be expected would be about 3 g DW cells /L (ie. 60 x 0.051). Therefore, for none of these major essential elements to be growth limiting, the medium should contain (at a minimal level) 0.34g N, 0.045g P and 0.005g Mg. Clearly, all the selected media formulations contain in excess of these estimated threshold levels of N, P and Mg. The fermentation time course of pH-stat STR batch fermentations with these selected media and 6% xylose (series B133 and B141) are shown in Figure 26 and the operational parameters are summarized in Table 23.

It should be noted that the observations with CSL as supplement need to be viewed in the light of the fact that the material used was not fresh. It had been stored in the refrigerator for several months and contained a high level of lactic acid (approx. 70g/L). We were unable to detect any lactic acid in media prepared with fresh CSL product. It is not known to what extent this elevated lactate may have affected the fermentation performance of the biocatalyst.

The values for ethanol yield are not corrected for dilution by the titrant and, as discussed earlier, the difference in ethanol yield between pH 6.3 and 7.0 is due in part to this dilution effect. However, even after adjusting the observed ethanol yield for dilution by alkali ($\times 1.06$), the yield at pH in each case falls below the target value of 0.46 g/g (Fig. 27). The unusually low value for carbon recovery in these experiments, however, is perhaps reason enough to suspect the accuracy of these yield values. With the exception of the defined salts based medium, all media formulations resulted in a final ethanol concentration that met the target value of 25 g/L. Likewise, only in the defined salts based medium did the biocatalyst fail to achieve the productivity goal of 0.52 g/L-h (Table 23).

From the relationship between xylose concentration and final cell density shown in Figure 2, it can be assumed that in none of the fermentations with 6% xylose is growth carbon limited. Therefore, in all these fermentations it can be assumed that some substance(s) is/are limiting growth. However, the low growth yields in experiment B141 (b, c and d) suggest "uncoupled" growth (Table 24 and Fig. 27). In the case of B141 c and B141d, although growth yield is depressed, the rate of xylose utilization is not proportionately decreased. It is interesting to note that, in these two cases, the specific rates of xylose utilization (q_s) are estimated to be significantly higher than for either the reference medium (B141a) or the semi-defined medium comprised of tap water, ammonium phosphate and Veeprax yeast extract (B141b) (Fig. 27). With B141b, the rate of xylose utilization seems to decrease in proportion to the decrease in growth yield, resulting in an unaltered specific activity (Fig. 27). Because of the uncertainty surrounding the accuracy of the yield, we have chosen to use the rate of xylose utilization as the more meaningful comparative parameter in this series of fermentations.

In the economic analysis of Grethlein and Dill (1993), which was based on a batch process using recombinant *E. coli* in a biomass hydrolysate supplemented with CSL, it was assumed that 40g ethanol could be derived from 80g sugars (equivalent to a process yield of 0.50 g/g or 98% conversion efficiency) and that the fermentation would be complete in 48h (equivalent to a Q_s value of 1.67 or a volumetric productivity of 0.84 g/L-h). In our experiment with 6% xylose and CSL supplementation, the yield (corrected for dilution but not for the contribution from fermentables in the CSL) was 0.43g/g (83.4% efficiency) (Table 24) - the average volumetric productivity was 0.60 g/L-h and the maximum productivity was 0.69 g/L-h (Table 23). Based on the final cell concentration of 1.25 g DW cells/L, this is equivalent to specific productivities of 0.48 (av.) and 0.55 g/g-h (max.). This calculation was made to provide a point of comparison to the value for maximum specific activity for xylose conversion by recombinant *E. coli* KO11 of 0.33 g/g-h that

was used by von Sivers *et al.* (1994) in another economic analysis which focused on the use of inorganics as nutrient supplements for large-scale production of cellulosic ethanol.

Economic impact of nutrient supplementation on cost of ethanol from xylose

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. Clearly, the laboratory reference medium (sLB) is off the scale in terms of economic feasibility. We estimated our cost for this medium to be Can.\$1.95/L. Even the medium based on Veeplex yeast extract (\$8/Kg) and ammonium phosphate (fertilizer grade), at an estimated cost of about \$40/m³, is well beyond economic feasibility (equivalent to cost of \$4.10/gal denatured ethanol). However, at a cost of \$50/ton of CSL (5.5¢/Kg), the cost of CSL supplementation of prehydrolysate at a rate equivalent to 7.7g/L is \$0.42/m³ 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/m³ in the prehydrolysate feed to the C₅ fermentor and assuming a xylose to ethanol conversion efficiency of 90% of theoretical maximum (equivalent to the NREL yield goal 0.46g/g) (4) and complete product recovery, the cost of using CSL at the lower rate of 7.7kg/m³ would be 1.13¢/L or \$0.043 per US gal of E95 fuel ethanol. This cost estimate compares favorably with the value of \$0.042/gal estimated by Grethlein and Dill (1993) 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.* (1994) 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.* (1994) 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 of 42.7 kg/m³ (also containing 18 kg/m³ glucose) and estimated the cost of their inorganic supplementation to be \$192,000 per year (based on a plant producing at total of 6.9MML of E95 fuel ethanol per year). This is equivalent to a nutrient supplement cost of 2.78¢/L or \$0.106 per US gallon of denatured ethanol. von Sivers *et al.* (1994) 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 to minimise 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 (McMillan, 1994) and were estimated at 22% of total production cost (von Sivers *et al.*, 1994)

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.* (1993) 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. Recombinant *E. coli* KO11 was selected on the basis of its resistance to chloramphenicol. Even with a stability that would sustain 3 cycles in the absence of antibiotic, it is estimated that the cost for antibiotic required solely for seed culture propagation would not be insignificant¹. Setting aside any possible regulatory obstacles in terms of employing a genetically engineered biocatalyst, it can be concluded that the cost of antibiotic for the purpose of ensuring and maintaining high-performance of the biocatalyst, is as important a consideration as the cost effectiveness of nutrient supplementation.

¹ Ref: Lawford, H.G. & Rousseau, J. D. in: Proceedings of 17th Symposium on Biotechnology for Fuels & Chemicals, Vail, Colorado, May, 1995 (Poster #78)

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TABLES

Table 1 Elemental composition of bacteria (% dry wt. basis)

Element	<i>E. coli</i> ¹	Bacteria ² gram-negative	Bacteria ³ (in general)	Microorganisms ⁴ (in general)
Carbon (C)	50		ca 50	45 - 55
Nitrogen (N)	15	12	10	6 - 14
Phosphorous (P)	3.2	1.5	2.0 - 3.0	1 - 3
Sulphur (S)	1.1	0.33	0.2 - 1.0	0.02 - 1.0
Sodium (Na)	1.3		0.5 - 1.0	
Potassium (K)	1.5	0.75	1.0 - 4.5	0.5 - 2.0
Magnesium (Mg)	0.5	0.19	0.1 - 0.5	0.1 - 1.0
Calcium (Ca)	1.0	0.10	0.01 - 1.1	+ others ca 1.0
Iron (Fe)	0.24	0.015	0.02 - 0.20	0.001 - 0.01
Manganese (Mn)	trace	0.005	0.001 - 0.01	0 - 0.005
Copper (Cu)	trace	0.001	0.01 - 0.02	0.0001 - 0.001
Others	trace	trace	trace	

¹ Demain and Solomon (1981)

² Pirt (1975)

³ Aiba *et al.* (1973)

⁴ Miller and Churchill (1981)

Table 2 Formulations of some of the complex media used for culturing *Escherichia coli*

Component (g/L)	Medium 52	Nutrient Broth	Luria Broth	mLB	LB 1065
Bacto Tryptone			10.0	2.5	10.0
Bacto Yeast Extract			5.0	2.5	5.0
NaCl			5.0		10.0
Bacto Peptone	10.0	5.0			
Beef Extract		3.0			

'Bacto' is a Trade Name of Difco Laboratories Ltd. (Detroit, MI)

mLB - modified LB (see this study; Grohmann *et al.*, 1994)

LB 1065 (Luria-Bertani Medium - see Atlas, 1993)

Table 3 NREL performance goals for xylose fermenting microorganisms¹

	Process Parameters		
	Yield (g/g)	Max. Ethanol % (w/v)	Vol. Productivity (g/L-h)
Target Value	0.46	2.5	0.52

¹ McMillan (1994)

Table 4 Yield of ethanol from the 'reference' medium (LB) without added xylose

Time (h)	mLB	LB	LB + 0.2% EtOH	LB + 0.3% EtOH	LB + Xyl	
					Xyl	EtOH
0	0	0	2.11	3.10	16.4	0
4	0.02	0.68	2.50	3.31	15.8	2.72
8	0.03	0.48	2.42	3.40	12.5	2.36
24	0.51	0.45	2.51	3.56	0	8.31
Δ	0.51	0.68	0.40	0.46		

* values are given as g/L The inoculation cell density was at an OD of about 0.5

Table 5 Effect of inoculum size and pH - a summary of product distribution and operational parameters

Expt. Code	Medium (modification)	Substrate			Substrate Use			Products			Productivity		Yield		% C Recov.
		[Xyl] g/l	Q _s g /L-h	max.Q _s g/L-h	[Cells] gDW/L	[EtOH] g/L	[Succ] mM	[Lac] mM	[HAc] mM	Q _p g/L-h	max.Q _p g/L-h	Y _{p/s} g/g	pH		
B104b	LB	45.1	1.80	2.24	2.31	18.95	29.3	0.0	1.3	0.76	1.91	0.42	7.0	96	
B111a	sLB	42.4	1.93	2.29	2.28	20.52	31.2	0.0	13.2	0.93	1.25	0.48	7.0	113	
B111b	DS + 0.5 LB	41.8	1.74	2.00	2.08	20.82	24.4	0.0	8.7	0.87	1.15	0.50	7.0	112	
B133a	mLB	42.1	1.28	1.96	1.88	20.06	20.2	0.1	0.8	0.61	0.78	0.48	7.0	104	
B142a	sLB pH 6.0	45.1	1.07	1.67	1.74	22.03	15.6	0.0	0.0	0.52	0.82	0.49	6.0	104	
B142b	sLB pH 6.3	43.6	1.15	2.14	1.78	19.89	21.8	0.0	9.4	0.52	0.86	0.46	6.3	102	
B142c	sLB pH 6.6	40.8	1.23	1.80	1.84	18.30	19.9	0.0	11.5	0.56	0.79	0.45	6.6	102	
B142d	sLB pH 7.0	42.6	1.18	1.58	2.01	18.56	15.5	0.0	10.5	0.52	0.66	0.44	7.0	96	
B041	sLB 0.04 gDW cells/L	33.0	1.65	1.65	1.93	15.08	11.0	0.0	13.3	0.76	0.76	0.46	7.0		
B041d	sLB 0.14 g	36.1	1.90	1.90	2.15	16.08	9.3	0.0	0.0	0.86	0.86	0.45	7.0		
B041e	sLB 0.40 g	33.8	2.25	2.25	2.48	15.18	9.3	0.0	16.3	1.01	1.01	0.45	7.0		
B041f	sLB 1.20 g	29.0	2.60	2.60	3.28	14.38	0.0	0.0	0.0	1.30	1.30	0.50	7.0		
B041g	sLB 3.00 g	28.0	2.80	2.80	4.39	13.88	0.0	0.0	0.0	1.40	1.40	0.50	7.0		

LB = Luria Broth

sLB = LB supplemented with 0.5 mM Mg + 17 mM PO₄

mLB = 2.5 g/L Yeast Extract + 2.5 g/L Tryptone

Expt. code (B) = pH-stat STR batch fermentation

Note: ethanol yield for reference LB (B104b) may be low because 2N alkali was used as titrant and no account was made for dilution in calculating yield

Table 6 Standard fermentation conditions

	Flask	Stirred Tank
Vessel Type	125ml capped Erlenmeyer	NBS MultiGen™ F2000 (2L)
Volume Medium	100ml	1500ml (total)
Xylose conc'n	ca 20 g/L	ca 40 g/L
Inoculum (OD)	0.1 - 0.2	0.1 - 0.2
Anaerobiosis	small head space/capped not flushed with N ₂	autoclaved medium/sealed headplate head space not flushed with N ₂
Agitation	shaking at ca 100RPM	Rushton turbine impellers (3) agitation at ca 100RPM
Temperature	water bath at 30°C	water circulation at 30°C
pH	0.1M P _i buffer	7.0 +/- 0.1
Titrant	initially adjusted to 7	4N KOH

Table 7 Listing of the commercial products tested as substitutes for Difco Yeast Extract and Tryptone

Product	Type	Manufacturer
Veeprex B430	yeast extract	Champlain Industries Ltd.
Veeprex B800	yeast extract	Champlain Industries Ltd.
Tureen AYP-65	yeast extract	Champlain Industries Ltd.
Yeast Extract	yeast extract	Gibco
Yeast Extract	tech. grade	Difco Laboratories Ltd.
Casamino Acids	casein digest	Difco Laboratories Ltd.
Pancase S	casein digest	Champlain Industries Ltd.

These products were screened initially in flask fermentations (see F014)

Table 8 Nitrogen content of complex culture medium components

Source of N	% N	Min. Amount (g/L)	
		Required to Support	
		2 gDW cells/L	3 g DW cells/L
Nitrogen (N)	100.0	0.228	0.342
NH ₄ Cl	26.0	0.88	1.35
(NH ₄) ₂ HPO ₄	21.2	1.08	1.61
Glutamic acid	9.3	2.45	3.69
Bacto Casamino Acids	10.0	2.28	3.42
Bacto Yeast Extract	9.18	2.48	3.73
Veeprex B430	8.5	2.68	4.02
Bacto Tryptone	13.14	1.74	2.60
Pancase S	13.0	1.75	2.63

Table 9 The nitrogen content of different complex and semi-defined media formulations

Medium Composition	N (g/L)
LB (sLB)	1.77
mLB	0.56
DS	0.61
DS + 2.5 DYE	0.84
DS + 0.625 DYE	0.68
DS + 2.5 VYE	0.82
DS + 2.5 VYE + 1 P	0.95
TW + 2.5 VYE + AP	0.51
TW + 2.5 VYE + AP + 1 P	0.64

Abbrev: DS, Defined salts medium (MM8)

DYE, Difco Yeast Extract

VYE, Veeprex B430; P, Pancase S

AP, (NH₄)₂HPO₄ added at 1.4 g/L

Table 10

Semi-defined media - a summary of product distribution and operational parameters

Expt. Code	Medium (modification)	Substrate			Substrate Use					Products			Productivity		Yield	
		[Xyl] g/l	Q _s g /L-h	max.Q _s g/L-h	[Cells] gDW/L	[EtOH] g/L	[Succ] mM	[Lac] mM	[HAc] mM	Q _p g/L-h	max.Q _p g/L-h	Y _{p/s} g/g	pH	% C Recov.		
B111a	sLB (reference)	42.4	1.93	2.29	2.28	20.52	31.2	0.0	13.2	0.93	1.25	0.48	7.0	113		
B111b	DS + 0.5 LB	41.8	1.74	2.00	2.08	20.82	24.4	0.0	8.7	0.87	1.15	0.50	7.0	112		
B133a	mLB	42.1	1.28	1.96	1.88	20.06	20.2	0.1	0.8	0.61	0.78	0.48	7.0	104		
B111c	DS + 2.5 DYE	49.3	1.50	1.80	2.06	21.92	31.2	0.0	11.4	0.66	0.75	0.44	7.0	101		
B113a	DS + 0.625 DYE	47.4	1.18	1.52	1.20	19.65	30.1	0.0	11.2	0.49	0.70	0.42	7.0	94		
B113d	DS + 5.0 VYE	48.6	1.35	1.98	1.38	20.91	33.1	0.0	13.0	0.58	0.86	0.43	7.0	98		
B111d	DS + 2.5 VYE	50.0	0.89	1.01	1.20	22.03	30.4	0.0	14.9	0.39	0.52	0.44	7.0	99		
B113c	DS + 2.5 VYE + 1P	48.7	1.43	1.83	1.42	21.25	29.6	0.0	9.0	0.62	1.00	0.44	7.0	96		
B114a	TW + AP + 2.5VYE	39.9	1.33	1.63	1.45	18.41	33.7	0.0	14.4	0.61	0.85	0.46	7.0	108		
B114b	TW + AP + 1P	41.7	1.30	1.64	1.39	18.00	33.4	0.0	14.2	0.56	0.72	0.44	7.0	101		
B114c	TW + AP + 2.5VYE + 1P	40.4	1.44	1.66	1.58	17.35	38.9	0.0	15.9	0.62	0.68	0.43	7.0	104		
B115a	" (+ Mg)	41.7	1.35	1.74	1.32	18.98	34.5	0.0	12.4	0.61	0.80	0.45	7.0	105		

LB = Luria Broth

sLB = LB supplemented with 0.5 mM Mg + 17 mM PO₄

mLB = 2.5 g/L Yeast Extract + 2.5 g/L Tryptone

DS = Defined salts medium (MM8)

TW = Toronto Tap Water

DYE = Difco Yeast Extract (g/L)

VYE = Veeprax B430 Yeast Extract (g/L)

T = Bacto Tryptone (g/L)

P = Pancase S (g/L)

AP = Ammonium phosphate

Table 11 **Estimated cost of different media formulations**

Medium	Cost (\$/L)
LB	1.95
DS + VYE + P	0.34
TW + VYE + AP + P	0.10
DS	0.28

Table 12 Composition of corn steep liquor

Component (weight %)	Ref. 1	Ref. 2
Solids	40-50	50
Carbohydrate		5.8
Protein	25-40	24
Amino acids		
Arginine		0.4
Cystine		0.5
Glycine		1.1
Histidine		0.3
Isoleucine		0.9
Leucine		0.1
Lysine		0.2
Methionine		0.5
Phenylalanine		0.3
Threonine		
Tryptophan		
Tyrosine		0.1
Valine		0.5
Fat	0	1.0
Fibre	0	1.0
Vitamins (mg/100g)		
Inositol	602	
Choline	351	
Niacin (B6)	8.4	
Pantothenic acid	1.5	
Pyridoxine	0.9	0.2
Thiamine (B1)	0.3	0.09
Biotin	0.03	0.09
Minerals (total ash)	8.0	8.8
K, P, Mg, Ca, S, Na, Fe, Zn, Mn, Cu, Cr, Mo, Se, Co		

(1) Anon (1975) "Properties and Uses of Feed Products from Corn Wet-Milling Operations." Corn Refiners Association Inc., Washington, DC

(2) Zabriskie, D. W.; Armiger, W. B. ; Phillips, D. H and Albano, P. A. (1982) Traders Guide to Fermentation Media Formulation. Traders Protein, Memphis, TN

Table 13 Amounts of CSL used in media formulations and corresponding estimated contribution to total nitrogen

CSL (mL)	solids (g)	total N (g)
55	23.4	1.55
37	15.7	1.04
18	7.65	0.51
13.5	5.13	0.38
9	3.83	0.25
4.5	1.91	0.12

Table 14 CSL based media - a summary of product distribution and operational parameters

Expt. Code	Medium (modification)	Substrate			Substrate Use					Products			Productivity		Yield	
		[Xyl] g/l	Q _S g /L-h	max.Q _S g/L-h	[Cells] gDW/L	[EtOH] g/L	[Succ] mM	[Lac] mM	[HAc] mM	Q _p g/L-h	max.Q _p g/L-h	Y _{p/s} g/g	pH	% C Recov.		
B111a	sLB (reference)	42.4	1.93	2.29	2.28	20.52	31.2	0.0	13.2	0.93	1.25	0.48	7.0	113		
B121a	TW + 55ml CSL	36.3	1.45	2.00	1.40	20.22	28.4	0.0	18.1	0.81	1.10	0.56	7.0	128		
B121b	TW + 37ml CSL	36.3	1.45	1.96	1.26	19.56	25.3	0.0	14.3	0.78	1.10	0.54	7.0	122		
B121c	TW + 18ml CSL	35.9	1.33	1.64	1.10	17.83	46.8	0.0	16.6	0.66	0.85	0.50	7.0	105		
B124a	TW + 18ml CSL	44.7	1.32	1.57	1.21	18.34	49.6	0.0	16.8	0.54	0.63	0.41	7.0	100		
B124b	TW + 13.5ml CSL	46.5	1.33	1.57	0.98	17.36	45.7	0.0	15.8	0.50	0.50	0.37	7.0	90		
B124c	TW + 9.0ml CSL	45.0	1.07	1.44	0.81	20.35	51.1	0.0	27.3	0.48	0.67	0.45	7.0	110		
B124d	TW + 4.5ml CSL	45.2	0.73	0.88	0.51	18.96	48.3	0.0	25.0	0.30	0.36	0.42	7.0	100		
F20(5-7)	TW + 55ml CSL	0				av. 0.11										
F18 (6)	TW + 18ml CSL	20.8	1.01		1.08	9.15				0.44		0.44				
F18 (7)	“ + V	22.0	1.05		1.12	9.46				0.45		0.43				
F18 (8)	“ + V + T	22.0	1.03		1.21	9.46				0.44		0.43				
F18 (9)	sLB (control)	22.2	1.20		1.32	10.43				0.56		0.47				

Table 15 Formulation of some selected defined *E. coli* media

Component (g/L)	R Med*	H&J*	Med 57	Med 63	M 9	MM 6	MM 8
KH ₂ PO ₄			3.0	13.6	3.0	2.72	2.72
K ₂ HPO ₄	3.0	13.6	7.0			3.48	3.48
Na ₂ HPO ₄ .7H ₂ O					12.8		
NaHCO ₃	8.0						
NH ₄ Cl					1.0	2.25	2.25
(NH ₄) ₂ SO ₄		2.5	1.5	2.0			
Mg SO ₄		0.1	0.1				0.1
MgSO ₄ .H ₂ O (mg/L)	9.9						
MgSO ₄ .7H ₂ O				0.20		0.20	
FeSO ₄ .7H ₂ O (mg/L)		0.50	0.50	0.50		0.50	
FeCl ₃ .6H ₂ O (mg/L)							7.0
CaCl ₂ (mg/L)			10.0				
CaCl ₂ .2H ₂ O (mg/L)	7.4						12.0
Thiamine (mg/L)	1.0					0.05	0.05
Citric Acid		2.1				0.21	0.21
NaCl					0.50	5.0	5.0
MnSO ₄ H ₂ O (mg/L)	0.9						9.9
Adenine sulfate (mg/L)	2.1						
Uracil (mg/L)	1.4						
Lysine			0.1				

* Amino acid supplements added

R Medium (Riesenberg's Medium) from Handbook of Microbiological Media (1993)

H&J Medium taken from Hernandez & Johnson (1967)

Medium 57 and Medium 63 taken from ATCC Culture Catalogue

Medium M 9 taken from Sambrook *et al* (1994)

MM 6 - defined salts medium described by Lawford & Rousseau (1991)

MM 8 - defined salts medium of this study

Table 16 Elemental composition of various *E. coli* defined media

Element (mM)	LB*	sLB*	Med 57	Med 63	M9	MM6	MM8	TW + AP
N	126	126	22	30	19	42	42	21.2
P	3	20	26	100	70	20	20	10.6
K	0.8	26	32	100	22	30	30	0.29 ^o
Na	98	98		112		85		0.54
Cl	87	87	0.82		36	45	127	0.71
Mg	0.25	0.75	0.83	0.80		1.66	1.66	0.35
Ca	0.30	0.30	0.41				0.05	1.00
Fe ²⁺ (µM)	38	38	1.8	1.8		0.002		
Fe ³⁺ (µM)						45		
S	1.8	1.8	12	16		1.66	1.66	1.43
Mn (µM)	3	3						0.04
Zn	0.01	0.01						0.23
Cu (µM)	4	4						1.36
Citric Acid					1.0	1.0		
Thiamine (mg/L)	1.0	1.0				0.05	0.05	

* Reference (complex) media LB and sLB are included for comparative purposes

^o KOH is used as titrant to control pH

TW = Toronto Tap Water (analysis given in Appendix B)

AP = Ammonium Phosphate as (NH₄)₂HPO₄ added at 1.4 g/L

Table 17 Composition of defined *E. coli* media relative to requirement for N, P and Mg based on cell composition

Element (g/L)	LB*	sLB*	Med 57	Med 63	M9	MM6	MM8	TW + AP
N (0.114)	1.76	1.76	0.31	0.42	0.27	0.59	0.59	0.30
P (0.015)	0.003	0.62	0.81	3.10	2.17	0.62	0.62	0.33
Mg (0.0015)	0.0061	0.018	0.020	0.019		0.040	0.040	0.009

* Reference (complex) media LB and sLB are included for comparative purposes

Bracketed values represent the minimal requirement per g DW cells/L based on cell composition (Pirt, 1975)

TW = Toronto Tap Water (analysis given in Appendix B)

AP = Ammonium Phosphate as (NH₄)₂HPO₄ added at 1.4 g/L

Table 18 Vitamin content of CSL and media supplements

Vitamins in Corn Steep Liquor		Vitamins in Medium Supplements		
		This study		
(mg/100g CSL)		(mg/L) ¹	(mg/L)	
meso-Inositol	602.0	Inositol.2H ₂ O	309.0	15.0
Choline	351.0	Choline		10.0
Niacin	8.4	Nicotinic acid	0.323	1.5
Pantothenic acid	1.5	Ca Pantothenate	0.107	0.15
Pyridoxine	0.9	Pyridoxine HCl	0.012	0.15
Thiamine	0.3	Thiamine HCl	0.048	0.02
Biotin	0.03	Biotin	0.013	0.01
		Folic acid	0.017	
Riboflavin	0.6	Riboflavin	0.103	0.10

¹ Hernandez and Johnson (1967)

Table 19 **Composition of amino acid supplements**

Amino acids	(mg per L) ¹	(mg per L) ²	This study (mg per L)	
			AA	AA+
D-Alanine	20.4			
L-Aspartic acid	84.9		50	50
L-Arginine HCl	56.9	125		50
L-Asparagine		180		50
L-Cysteine HCl	17.0			50
L-Glutamic acid HCl	179.1		250	250
L-Glutamine HCl	350.0	610		
Glycine	70.9	65		
L-Histidine HCl	19.2	55	50	50
L-Hydroxyproline	41.4			
L-Isoleucine	48.3	170		50
L-Leucine	73.0	230		50
L-Lysine	98.8	230		50
L-Proline	54.3	43		50
L-Methionine	18.9	73		50
L-Phenylalanine	33.2	125	50	50
L-Serine	39.6	240		50
L-Threonine	39.4	120		50
L-Tryptophan	7.7	35	50	50
L-Tyrosine	36.4	214	50	50
L-Valine	55.0	170		50

¹ Hernandez and Johnson (1967)

² R. M. Atlas (1993) "R Medium" in *Handbook of Microbiological Media*

Table 20 Defined media - a summary of product distribution and operational parameters

Expt. Code	Medium (modification)	Substrate			Substrate Use					Products				Productivity		Yield	
		[Xyl] g/l	Q _s g/L-h	max.Q _s g/L-h	[Cells] gDW/L	[EtOH] g/L	[Succ] mM	[Lac] mM	[HAc] mM	Q _p g/L-h	max.Q _p g/L-h	Y _{p/s} g/g	pH	% C Recov.			
B111a	sLB (reference)	42.4	1.93	2.29	2.28	20.52	31.2	0.0	13.2	0.93	1.25	0.48	7.0	113			
B107b	DS	29.8	0.21	0.24	0.58	11.56	0.0	0.0	10.4	0.08	0.18	0.39	7.0	81			
B110a	DS	40.5	0.34	0.36	0.44	17.00	15.9	0.0	18.0	0.14	0.18	0.42	7.0	92			
B110b	DS (LB inoc)	41.2	0.59	0.68	0.65	18.27	26.5	0.0	15.8	0.26	0.28	0.44	7.0	100			
F018(1)	DS - Th	8.0	0.17		0.60	2.12					0.04	0.26		61			
F018(2)	DS + V	16.1	0.33		0.90	5.82					0.12	0.36		77			
F019(2)	DS + V	19.3	0.44		1.48	7.81					0.18	0.40		103			
F019(3)	DS + V + 1G	18.9	0.47		1.25	8.58					0.21	0.45		112			
F019(1)	LB	19.0	0.59		1.64	9.07					0.28	0.48		117			
F024(1)	LB	21.9	0.99		1.40	10.61					0.48	0.48		103			
F024(2)	DS + V	22.0	0.61		0.83	9.51					0.26	0.43		97			
F024(3)	DS + V + 1.5G	21.1	0.58		0.83	8.80					0.24	0.42		94			
F024(4)	DS + V + 3.0G	21.8	0.51		0.76	8.72					0.20	0.40		90			
F024(6)	DS + V + 1 T	22.6	0.75		0.84	9.54					0.32	0.42		96			
B133b	DS + .5 Vit + 2.5T	42.2	1.03	1.35	1.82	18.97	22.9	3.5	11.9	0.46	0.65	0.45	6.3	103			
F025(1)	DS + V	17.0	0.35		0.84	7.46					0.16	0.44		109			
F025(2)	DS + .5V	15.8	0.33		0.84	6.82					0.14	0.43		106			
F025(3)	DS-Th + .5Thi/Nic	15.2	0.32		0.84	6.68					0.14	0.44		109			
F025(4)	DS + V + .25G	19.6	0.45		0.99	9.10					0.21	0.46		108			
F025(5)	DS + V + .5G	19.4	0.42		0.96	8.67					0.18	0.45		104			
F025(6)	DS (no N) + V + .5G	20.0	0.42		0.84	8.56					0.18	0.43		105			

continued.....

Table 20 (Continued) Defined media - a summary of product distribution and operational parameters

Expt. Code	Medium (modification)	Substrate			Substrate Use			Products			Productivity		Yield	
		[Xyl] g/l	Q _s g /L-h	max.Q _s g/L-h	[Cells] gDW/L	[EtOH] g/L	[Succ] mM	[Lac] mM	[HAc] mM	Q _p g/L-h	max.Q _p g/L-h	Y _{p/s} g/g	pH	% C Recov.
B111a	sLB (reference)	42.4	1.93	2.29	2.28	20.52	31.2	0.0	13.2	0.93	1.25	0.48	7.0	113
B134c	DS + .5 V + .25G	38.6	0.47	0.66	1.05	18.12	19.6	0.0	17.9	0.22	0.39	0.47	7.0	106
F026(1)	LB	21.5	0.86		1.88	10.81					0.43	0.50		120
F026(2)	DS + V .25G	19.6	0.46		1.13	9.24					0.22	0.47		106
F026(3)	DS-C + V + .25G + TE	21.0	0.47		1.27	9.93					0.22	0.47		105
F026(4)	DS + V + .25G + AA	21.6	0.50		1.24	10.41					0.24	0.48		108
B135c	DS + .5 V + .25G + AA	39.82	0.47	0.63	0.98	16.72	18.8	0.0	11.5	0.20	0.26	0.42	7.0	93
B135d	DS + .5 V + .25G + AA+	42.87	0.76	1.05	1.59	17.52	15.5	14.4	10.3	0.31	0.44	0.41	7.0	94

Codes for Table 20

- TW = Toronto Tap Water
- C = Citric acid
- Th = Thiamine
- Nic = Nicotinic acid
- AP = 1.4 g/L Ammonium monohydrogenphosphate
- CSL = Corn Steep Liquor (NACAN Products Ltd) (ml/L)
- V = Vitamin Stock (added at 10 ml/L) - see Table 18
- G = Glutamic acid (g/L)
- AA = Amino acid cocktail = 50 mg/L each of Aspartic acid, Tyrosine, Tryptophan, Phenylalanine & 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

B = pH-stat STR batch fermentation F = shake flask fermentation with buffered medium

Table 21 **Composition of salts supplement and trace metals mixture used by Guimaraes *et al.* (1992) in fermentations of whey by recombinant *E. coli* KO11**

Salts supplement (g/L)

ammonium sulphate	2.0
magnesium sulphate.7H ₂ O	0.2
dipotassium phosphate	0.7
monosodium phosphate	0.3
trace metal mix	5 ml
thiamine*	1 mg

* *“thiamine omission had little effect on this simplified medium and was also eliminated in subsequent studies”*

Trace metal mix (g/L)^o

disodium EDTA	5.0
zinc sulphate.7H ₂ O	0.22
calcium chloride	0.50
manganous chloride	0.50
ferrous sulphate	0.50
ammonium molybdate.4H ₂ O	0.10
cupric chloride	0.16
cobalt chloride	0.16

^o Ref: Traders Guide to Fermentation Media, Traders Protein

**Table 22 Composition of *E. coli* media (6% Xylose)
with respect to potential limiting elements**

Element (g/L)	sLB*	DS	DS + V +0.25G + AA ⁺	TW + AP + 5 VYE	TW + 37ml CSL
N (0.114)	1.76	0.59	0.78	0.72	1.04
P (0.015)	0.62	0.62	0.62	0.35	ND
Mg (0.0015)	0.018	0.040	0.040	0.012	0.105

* Reference (complex) media LB and sLB are included for comparative purposes
 Bracketed values represent the minimal requirement per g DW cells/L based on cell composition (Pirt, 1975)
 ND = not determined; DS = Defined salts medium (MM8) as per formulation in this study
 TW = Toronto Tap Water (analysis given in Appendix B); V = vitamin supplement as per Table 18
 AP = Ammonium Phosphate as (NH₄)₂HPO₄ added at 1.4 g/L; CSL = corn steep liquor (Nacan Products Ltd)
 VYE = Veeprax Yeast Extract; G = glutamic acid; AA+ = cocktail of 17 amino Acids as per Table 19

Table 23 Media with 6% xylose - a summary of product distribution and operational parameters

Expt. Code	Medium (modification)	Substrate		Substrate Use		Products				Productivity		Yield		% C Recov.
		[Xyl] g/l	Q _s g/L-h	max.Q _s g/L-h	[Cells] gDW/L	[EtOH] g/L	[Succ] mM	[Lac] mM	[HAc] mM	Q _p g/L-h	max.Q _p g/L-h	Y _{p/s} g/g	pH	
B133c	sLB (pH 6.3)	60.3	1.55	1.90	2.74	29.34	24.2	22.0	10.2	0.75	0.99	0.49	6.3	111
B133d	sLB + V	62.7	1.69	2.28	2.71	30.72	24.9	8.2	0.8	0.83	0.85	0.49	6.3	107
B141a	sLB (pH 7.0)	62.7	2.02	2.40	2.98	26.71	25.9	0.0	14.5	0.86	0.98	0.43	7.0	96
B141b	TW + AP + 5 VYE	63.9	1.21	1.40	1.70	24.93	51.4	0.0	24.6	0.55	0.75	0.39	7.0	92
B141c	DS + V + 0.25G + AA ⁺	62.27	0.67	1.45	0.98	23.88	36.8	0.0	25.0	0.26	0.36	0.38	7.0	88
B141d	TW + 37ml CSL	62.2	1.41	1.93	1.25	25.00	50.0	0.0	10.2	0.60	0.69	0.40	7.0	92

Table 24 Growth yield and specific rate of xylose utilization for recombinant *E. coli* in selected media with 6% xylose

		Xylose g/L	Biomass gDW/L	$Y_{x/s}$ g/g	max Q_s g/L-h	q_s g/g-h	obs. $Y_{p/s}$ g/g	pH
B133c	sLB (pH 6.3)	60.3	2.74	0.039°	1.90	0.69	0.49	6.3
B133d	sLB + V	62.7	2.71	0.039°	2.28	0.84	0.49	6.3
B141a	sLB (pH 7.0)	62.7	2.98	0.041°	2.40	0.81	0.43	7.0
B141b	TW + AP + 5 VYE	63.9	1.70	0.027	1.40	0.82	0.39	7.0
B141c	DS + V + 0.25G + AA ⁺	62.27	0.98	0.023	1.45	1.48	0.38	7.0
B141d	TW + 37ml CSL	62.2	1.25	0.020	1.93	1.54	0.40	7.0

$Y_{x/s}$ - growth yield co-efficient based on g DW cells per g xylose consumed (end-point)

° Growth yield ($Y_{x/s}$) for xylose corrected for contribution from LB

q_s - specific rate of xylose utilization determined as value of max Q_s divided by max. cell concentration

obs. $Y_{p/s}$ - yield was based on final EtOH and xylose added and was not corrected for dilution by KOH

Note: these parameters are compared graphically in Figure 27.

FIGURES

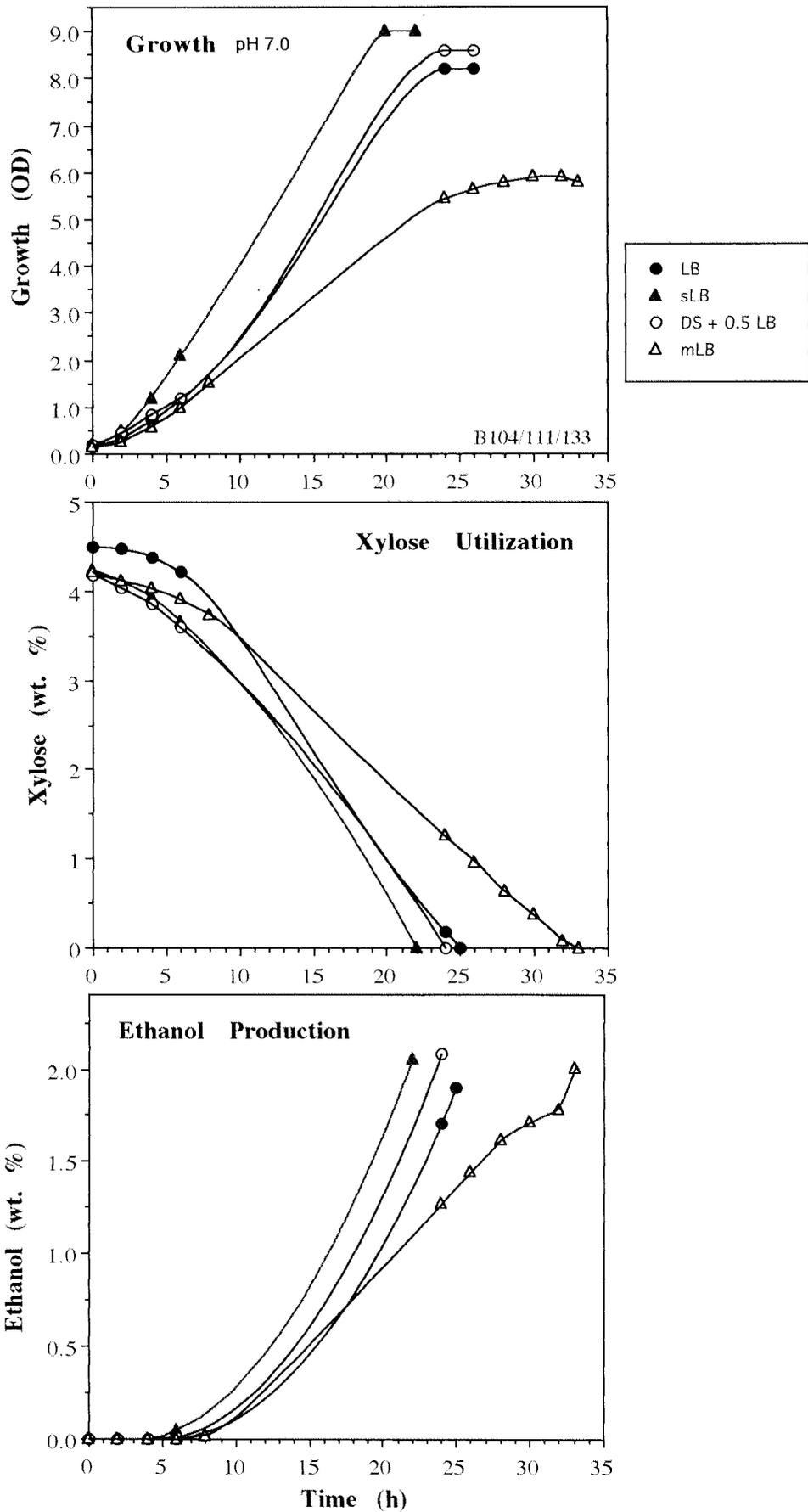


Figure 1 Time course of typical pH-stat STR batch fermentation with recombinant *E. coli* and "reference" medium (LB)

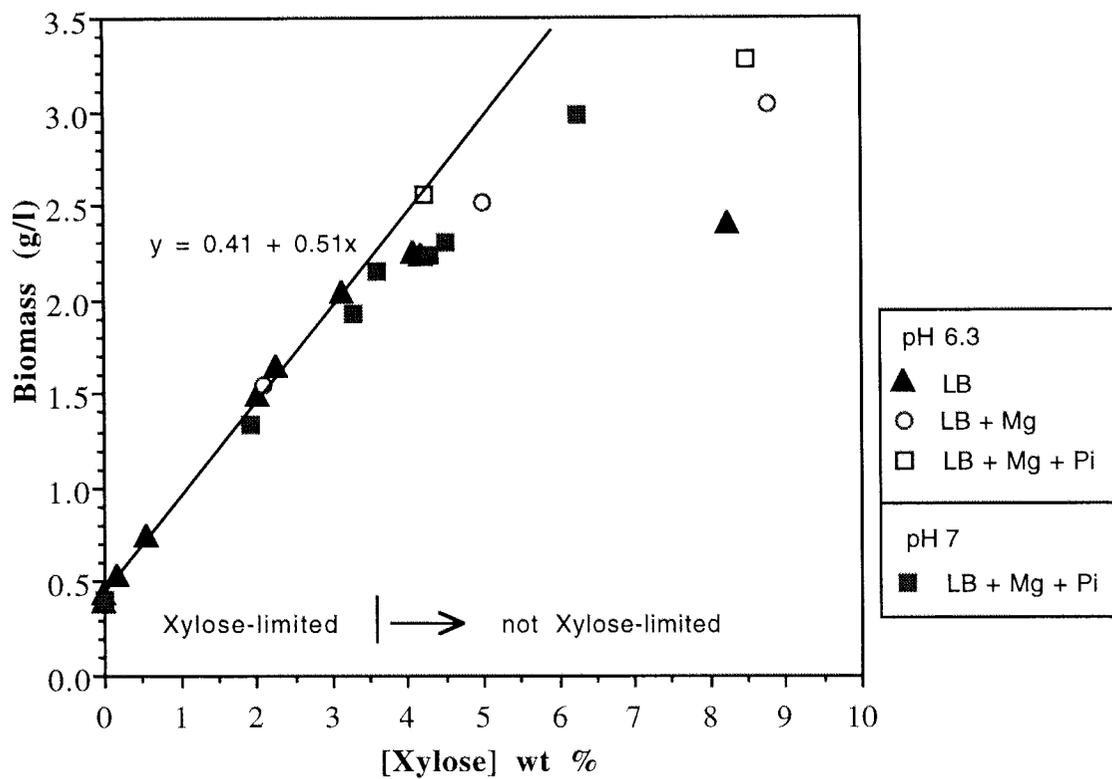


Figure 2 Final cell concentration as a function of xylose concentration

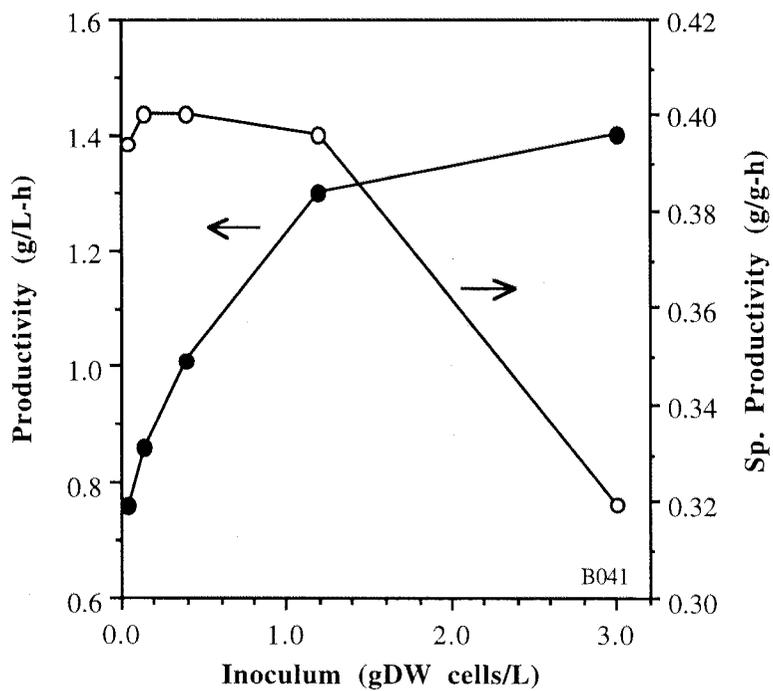


Figure 3 Productivity as a function of inoculum size

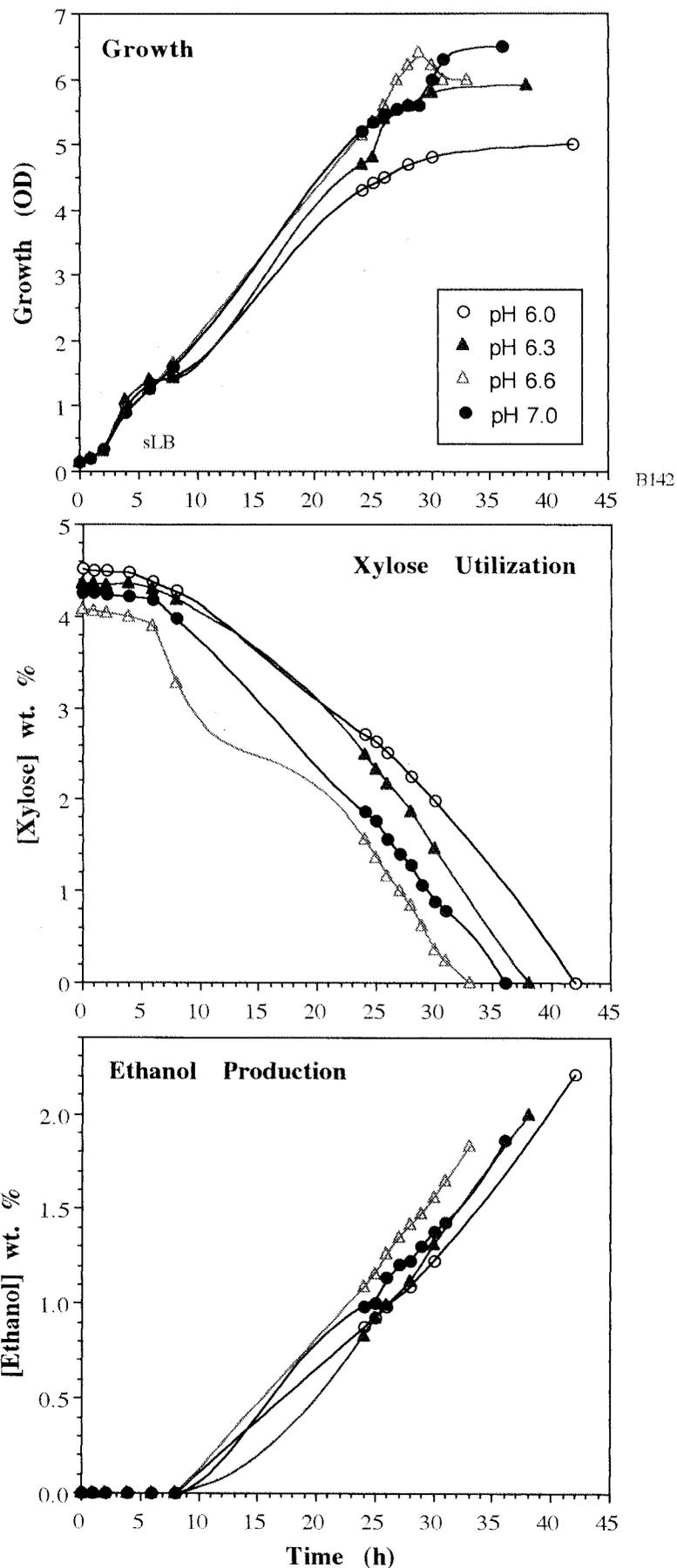


Figure 4 Effect of pH on growth and xylose fermentation

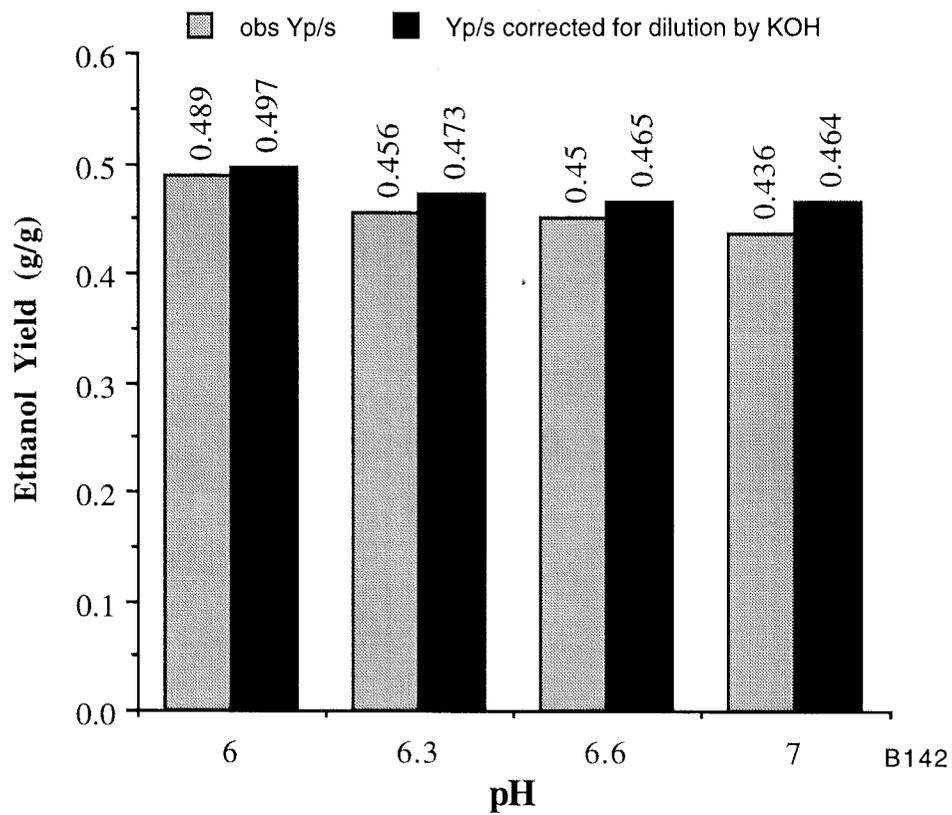


Figure 5 Ethanol yield as a function of pH and the dilution effect of titrant

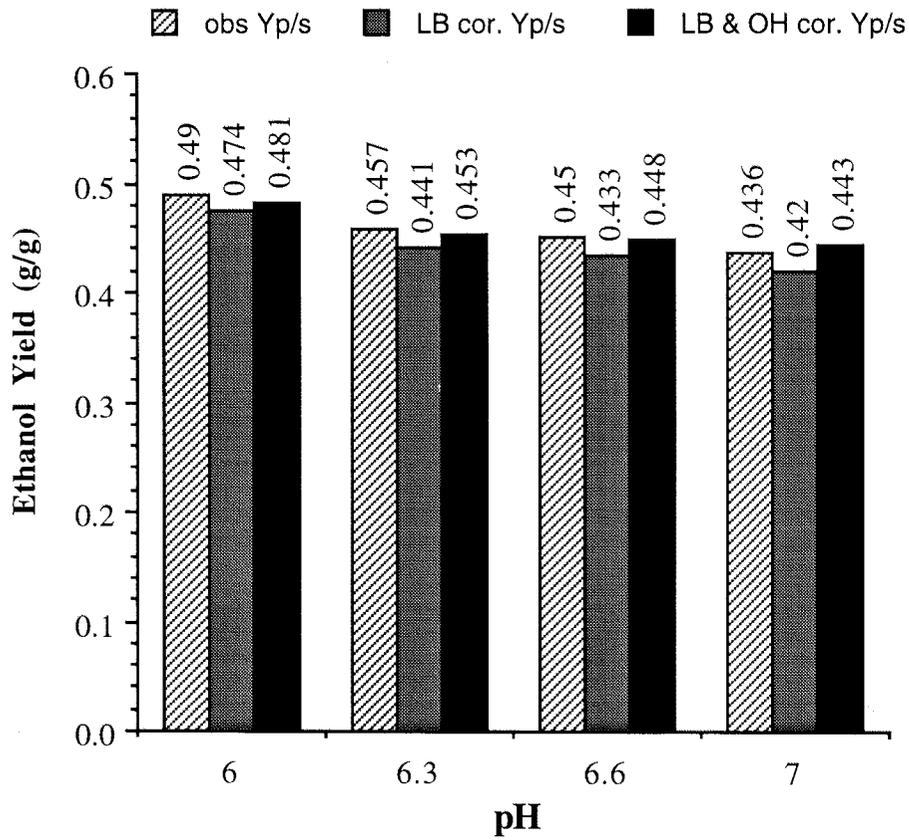


Figure 6 Correcting the observed yield for contribution by non sugar fermentables and dilution by titrant

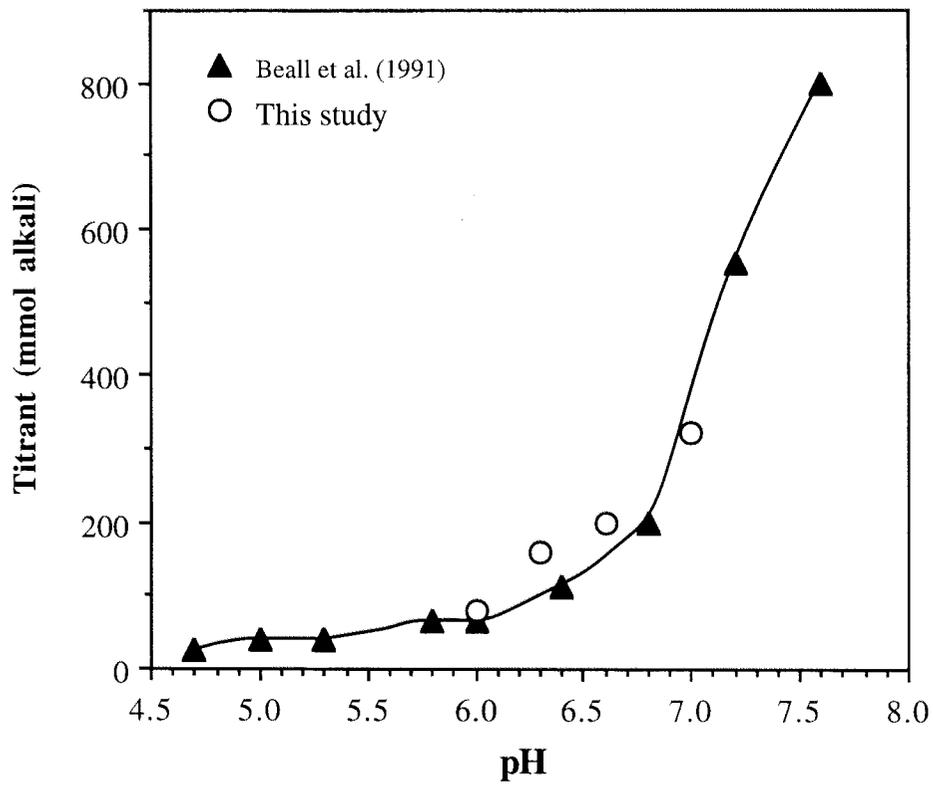


Figure 7 Amount of alkali titrant (mmoles) as a function of pH

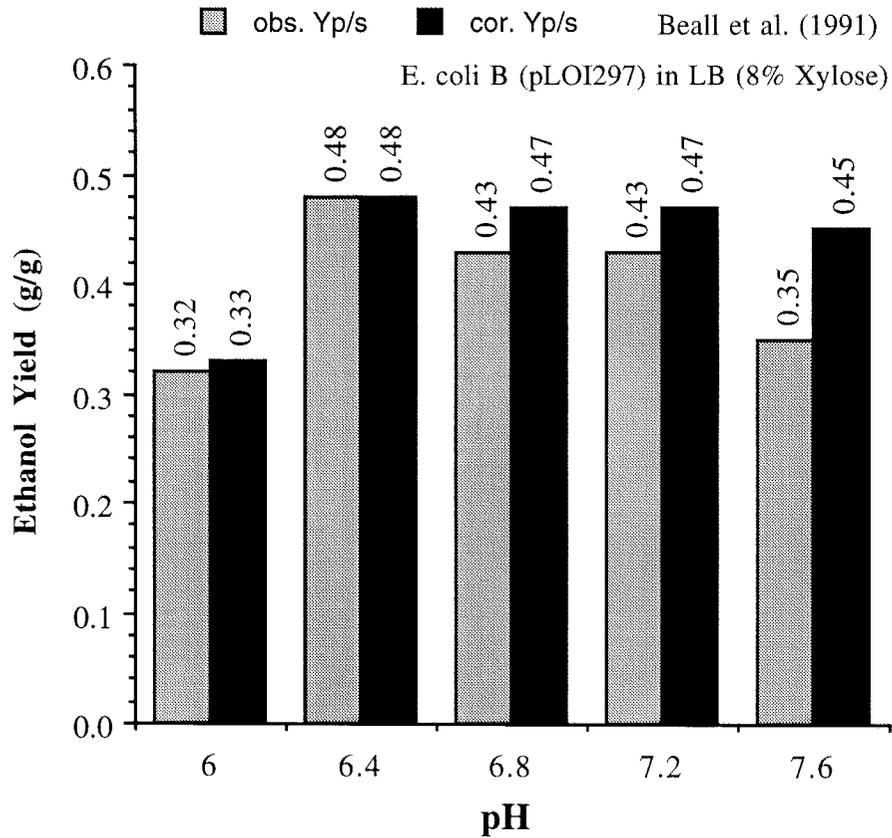


Figure 8 Observed and corrected ethanol yield as a function of pH
 Data taken from Table 1 of Beall *et al.* (1991)

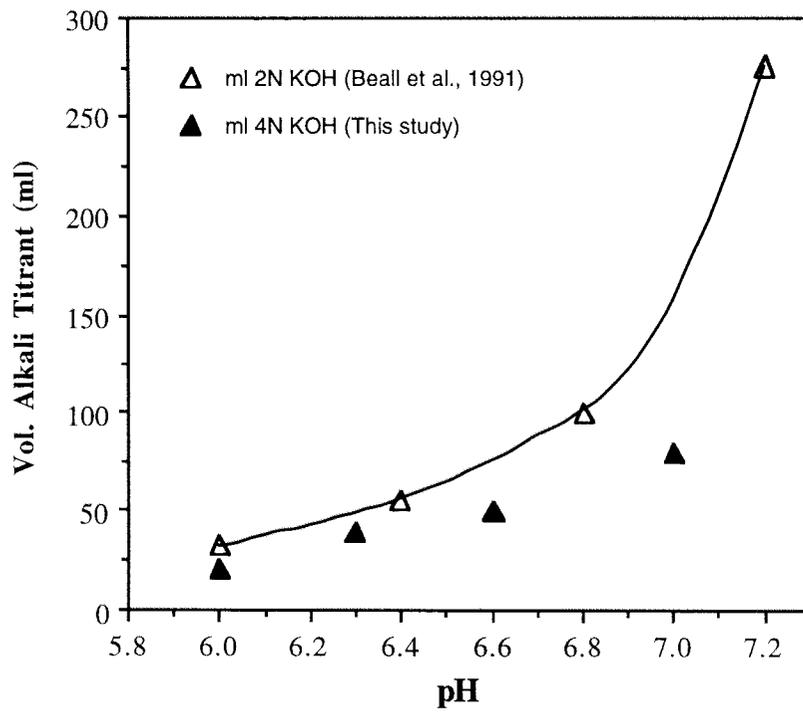


Figure 9 Volume of KOH added as a function of pH

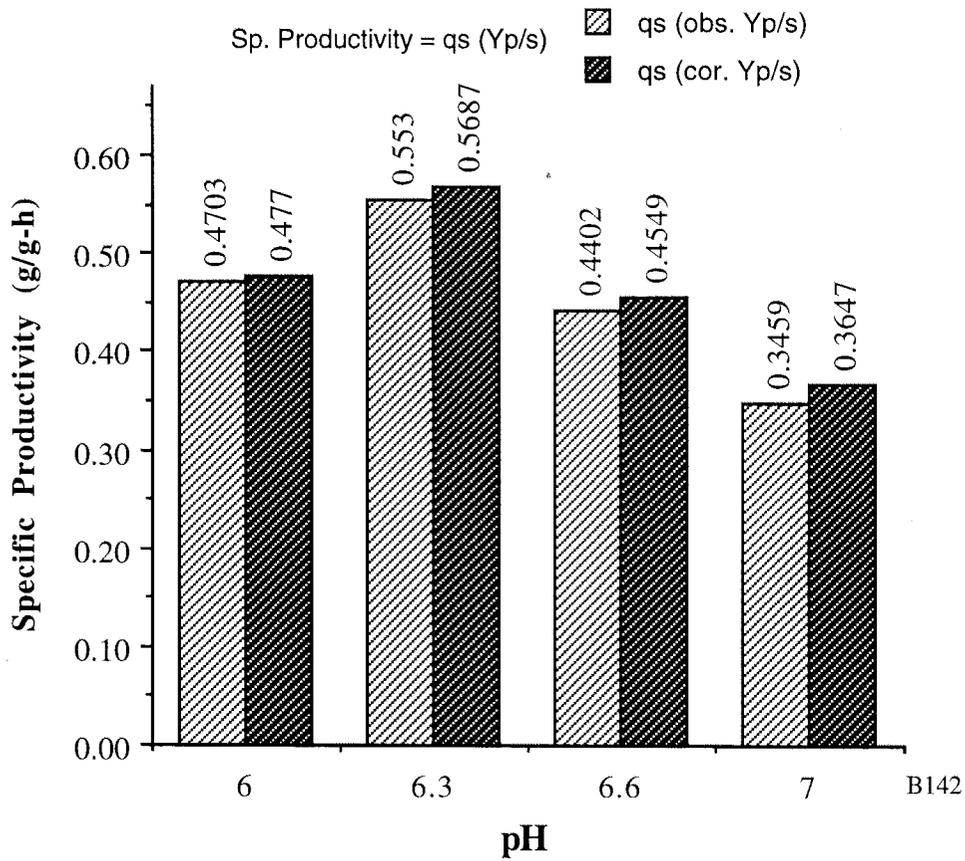


Figure 10 Effect of pH on specific productivity

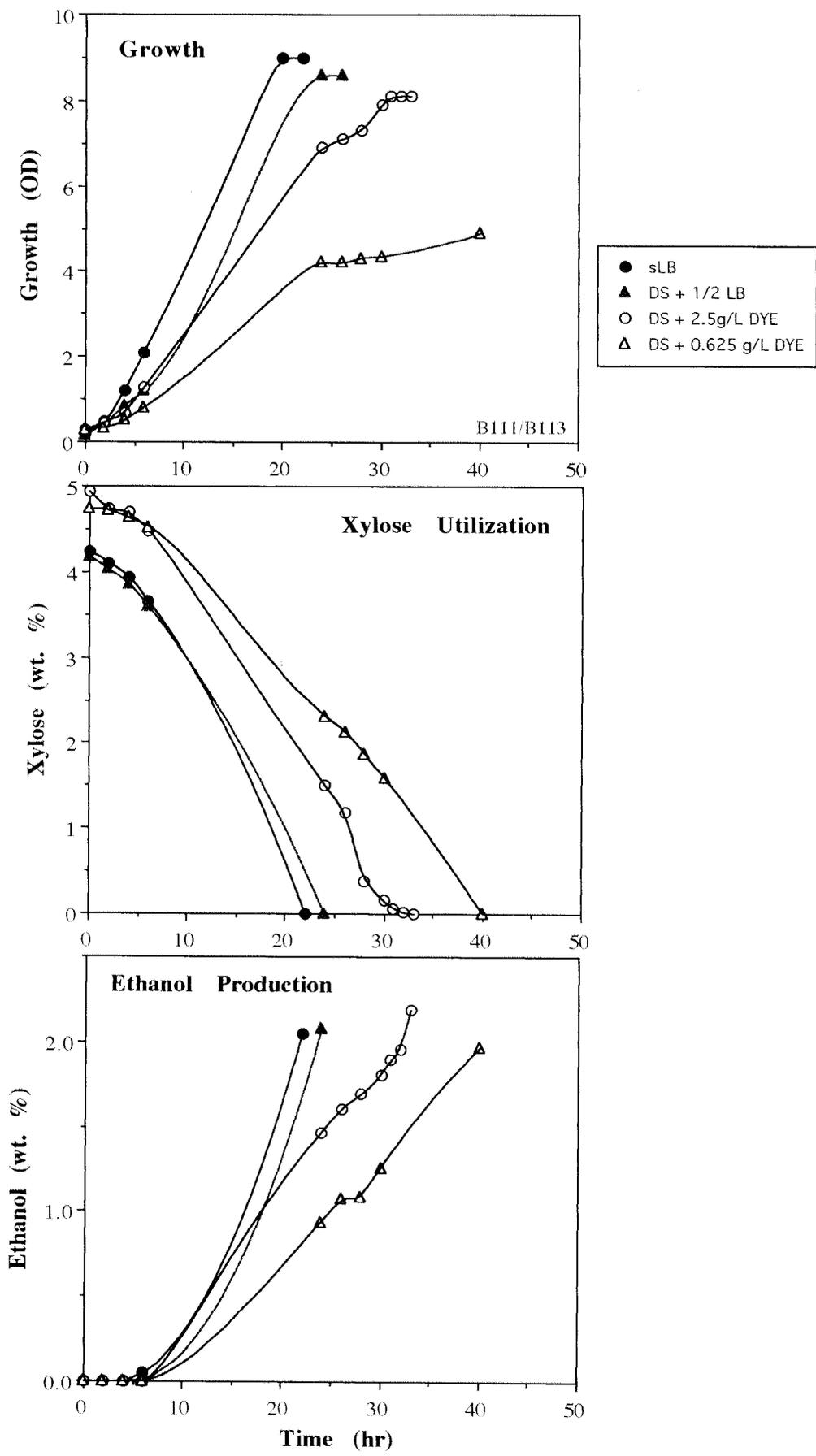


Figure 11 Effect of reduced amounts of Yeast Extract and Tryptone

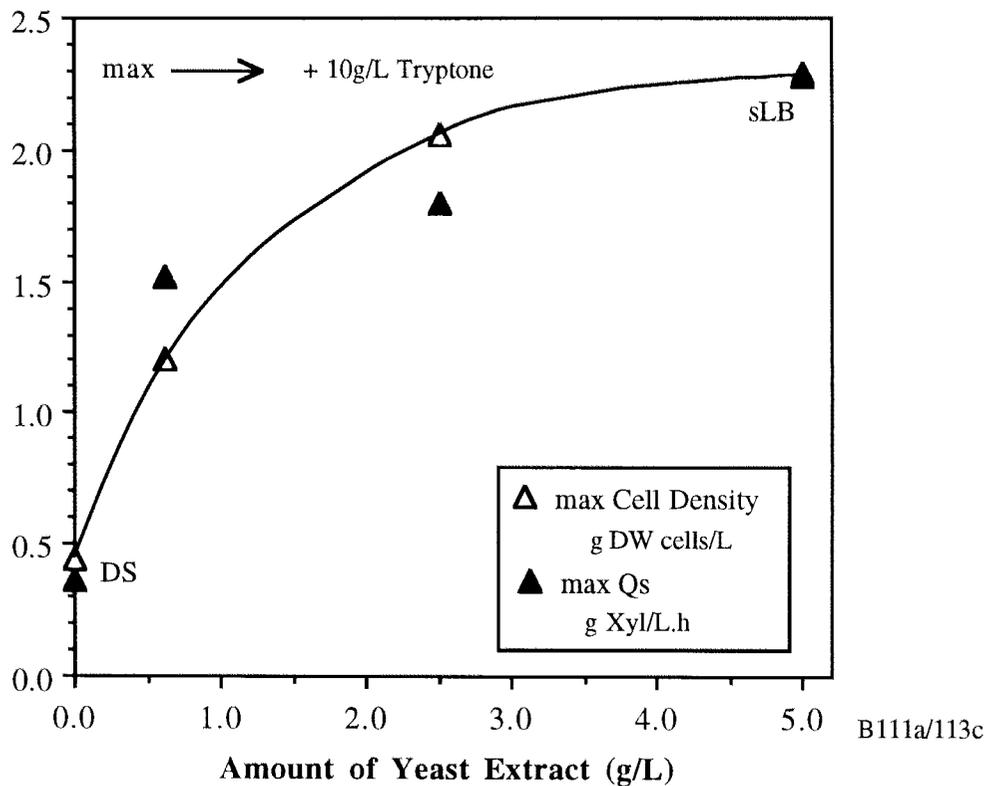


Figure 12 Effect of Difco Yeast Extract on cell concentration and rate of xylose utilization

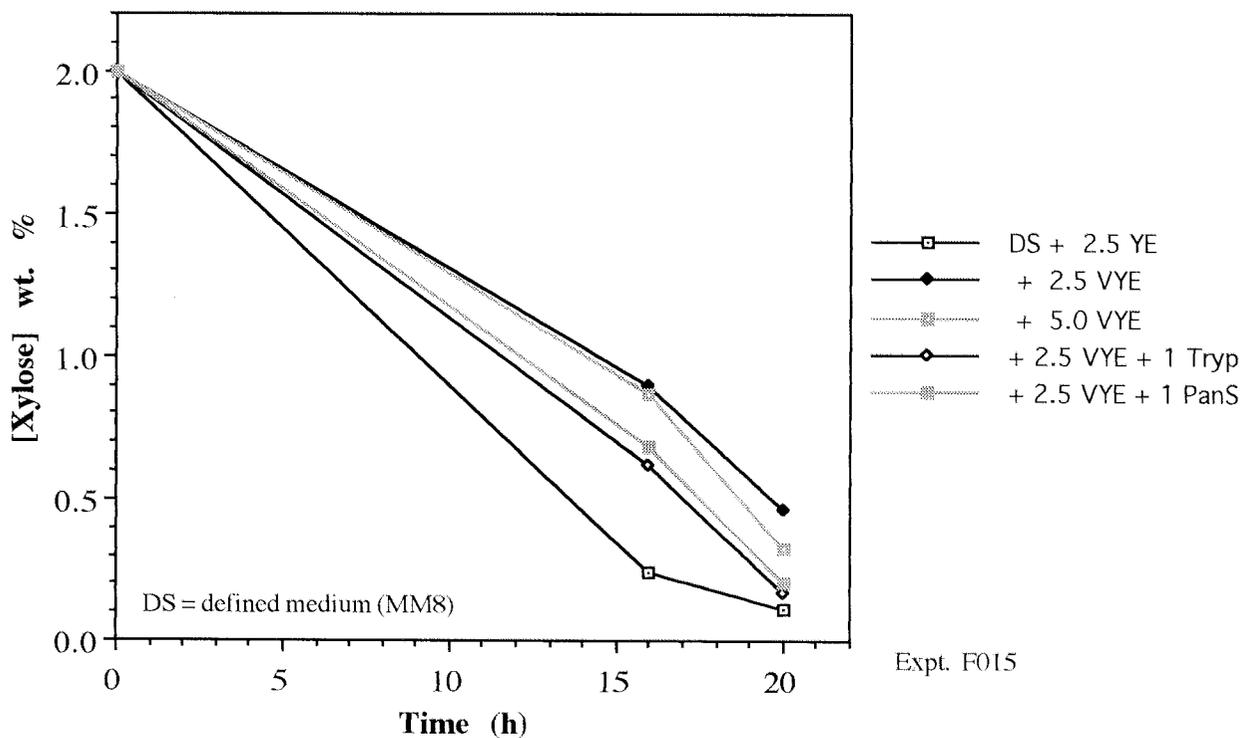
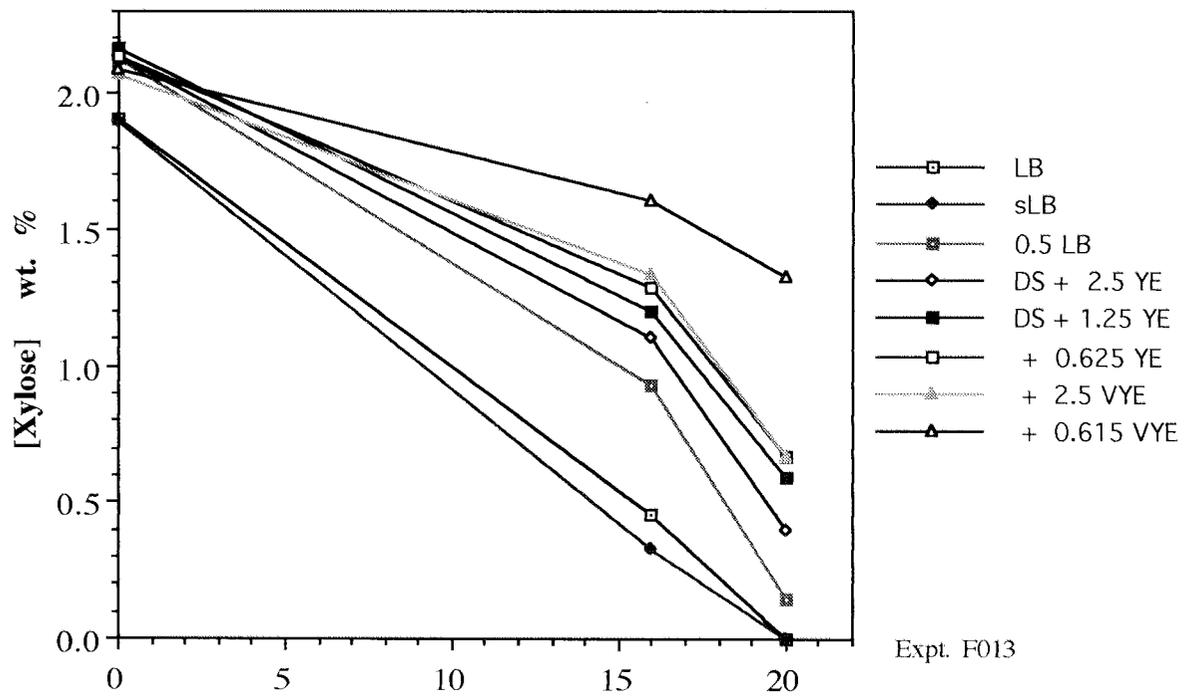


Figure 13 Time-course of flask fermentations with respect to xylose utilization - screening for the effect of Difco Yeast Extract "substitutes"

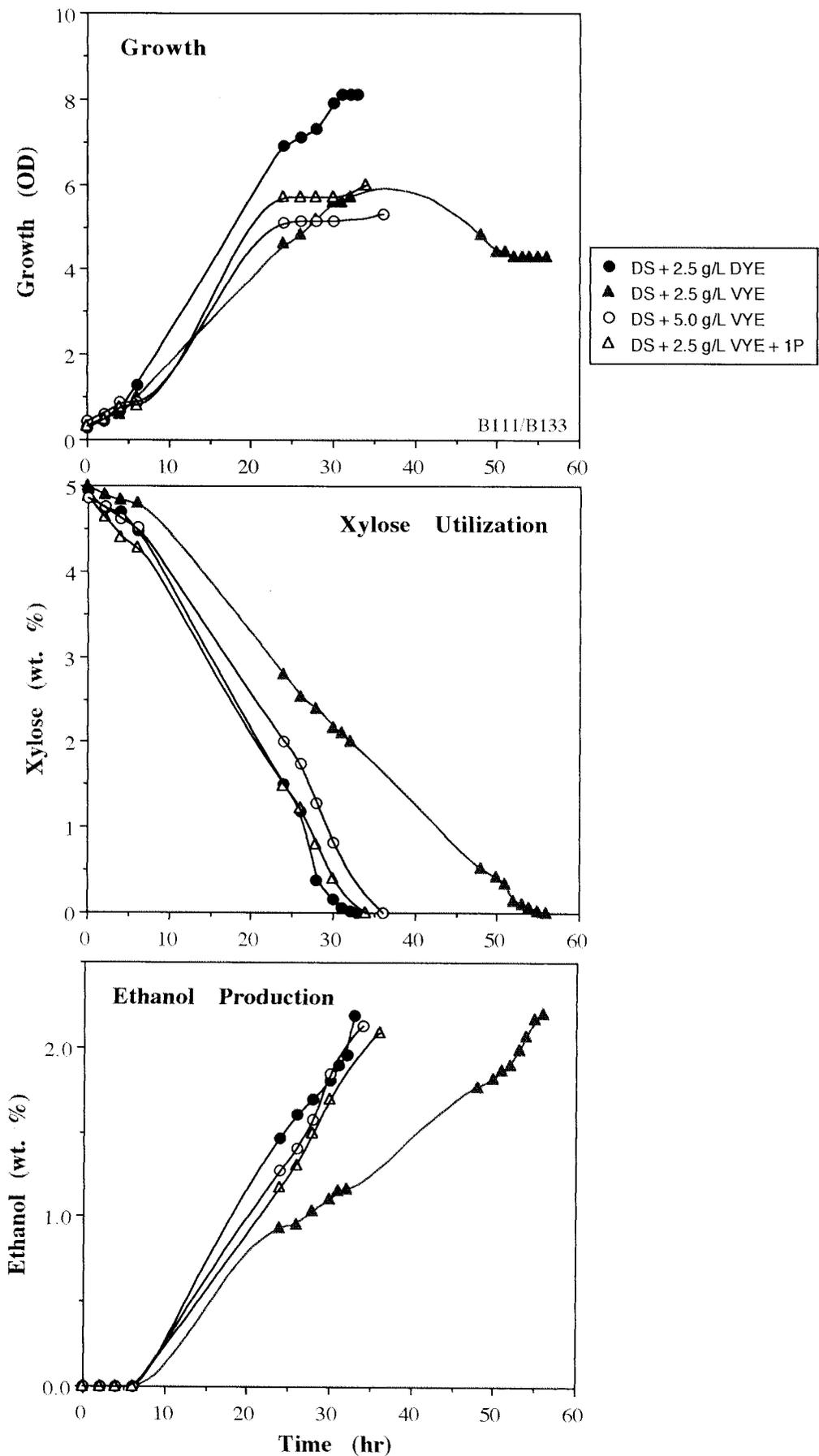


Figure 14 Effect of alternative sources of Yeast Extract and Tryptone

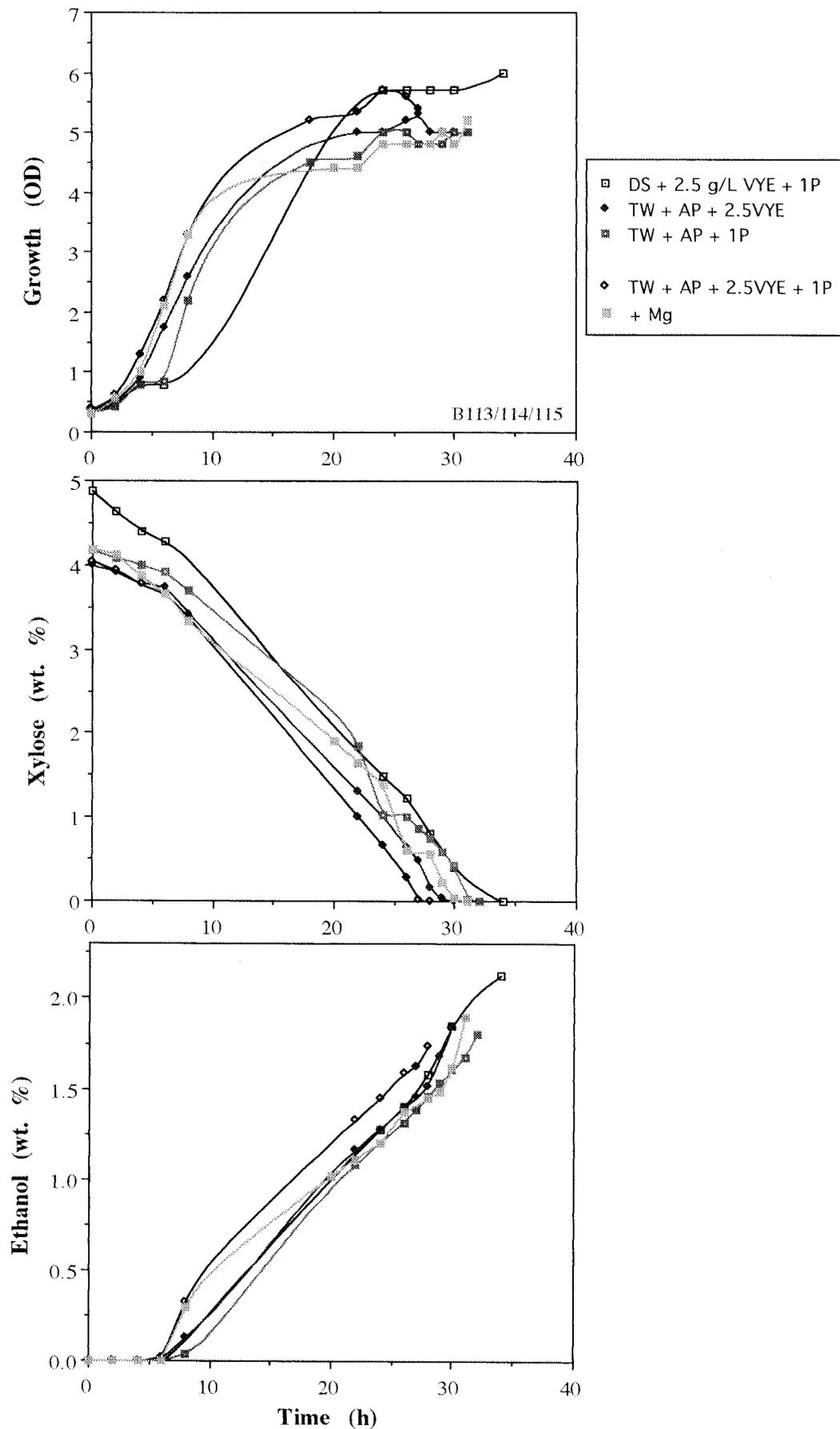


Figure 15 Effect of Yeast Extract and Tryptone "substitutes"

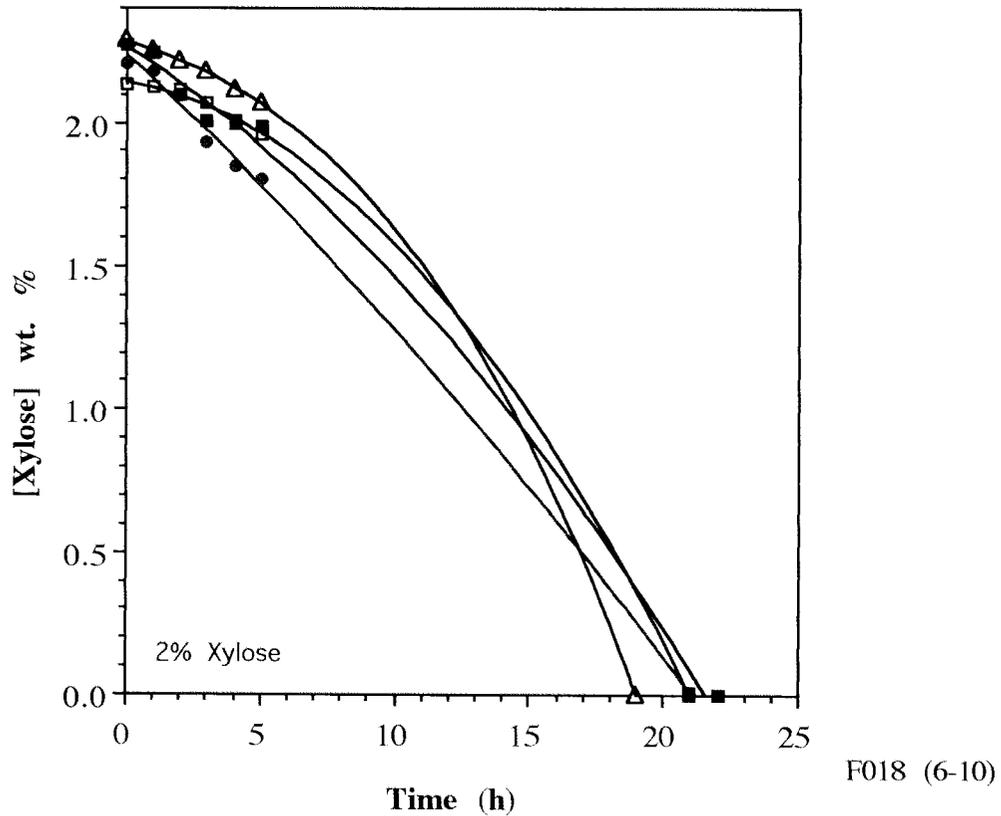
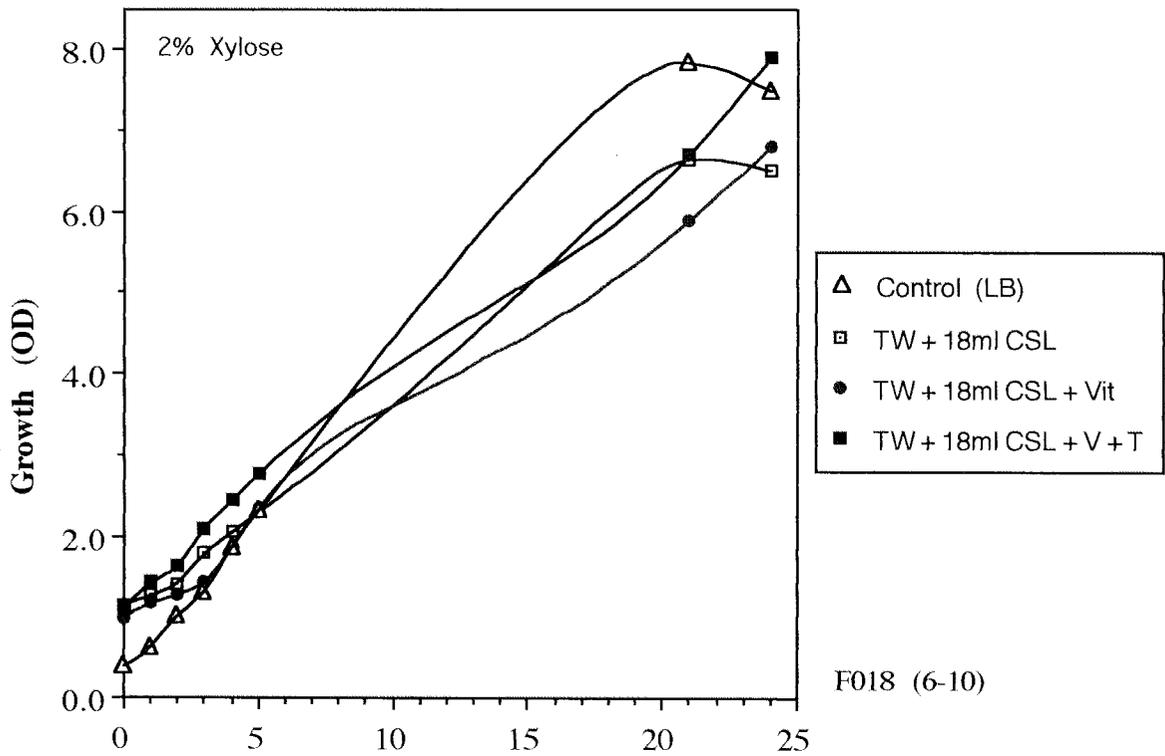


Figure 16 Effect of CSL supplementation with vitamins and tryptone

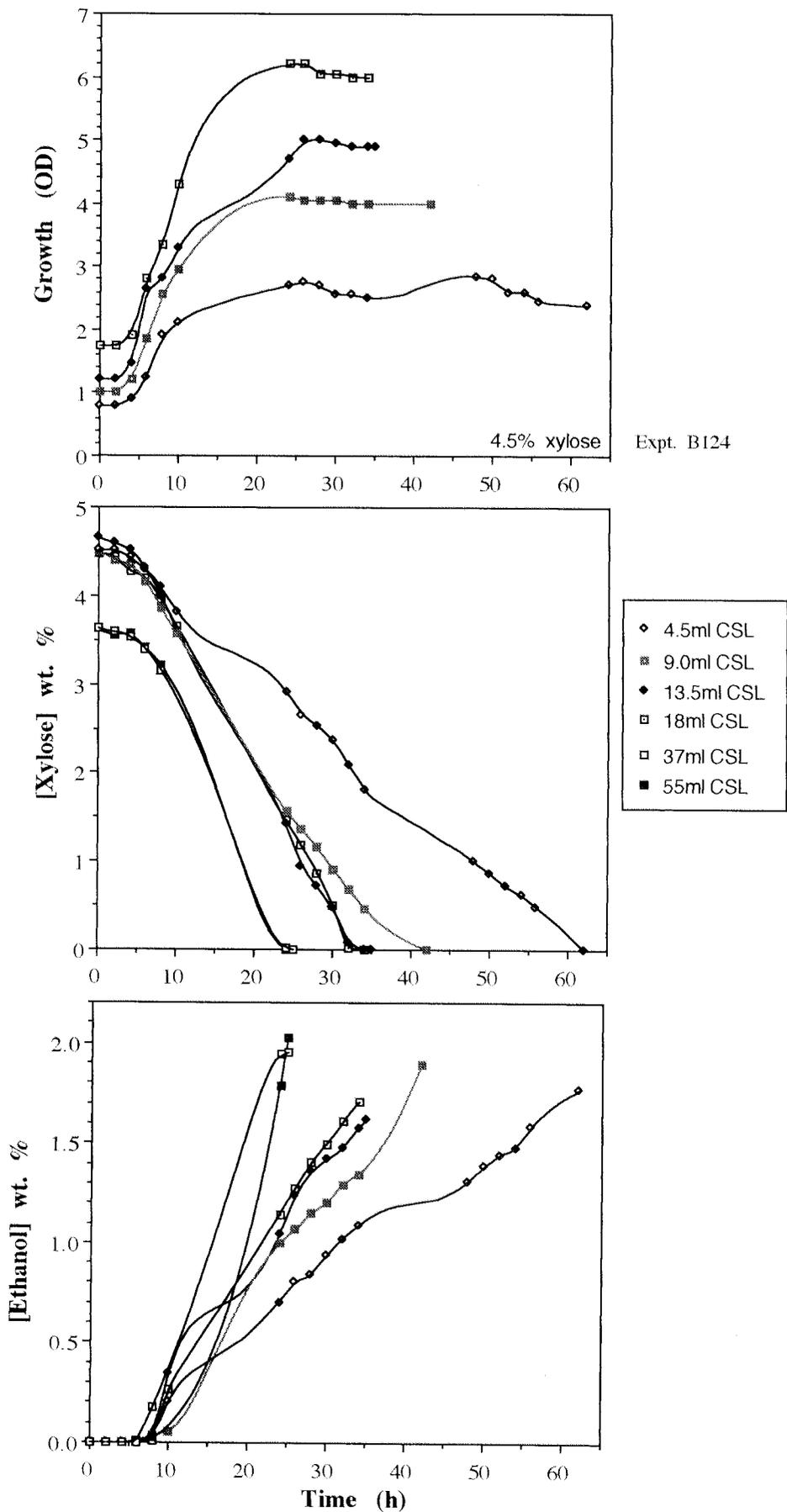


Figure 17 Effect of different amounts of CSL supplementation

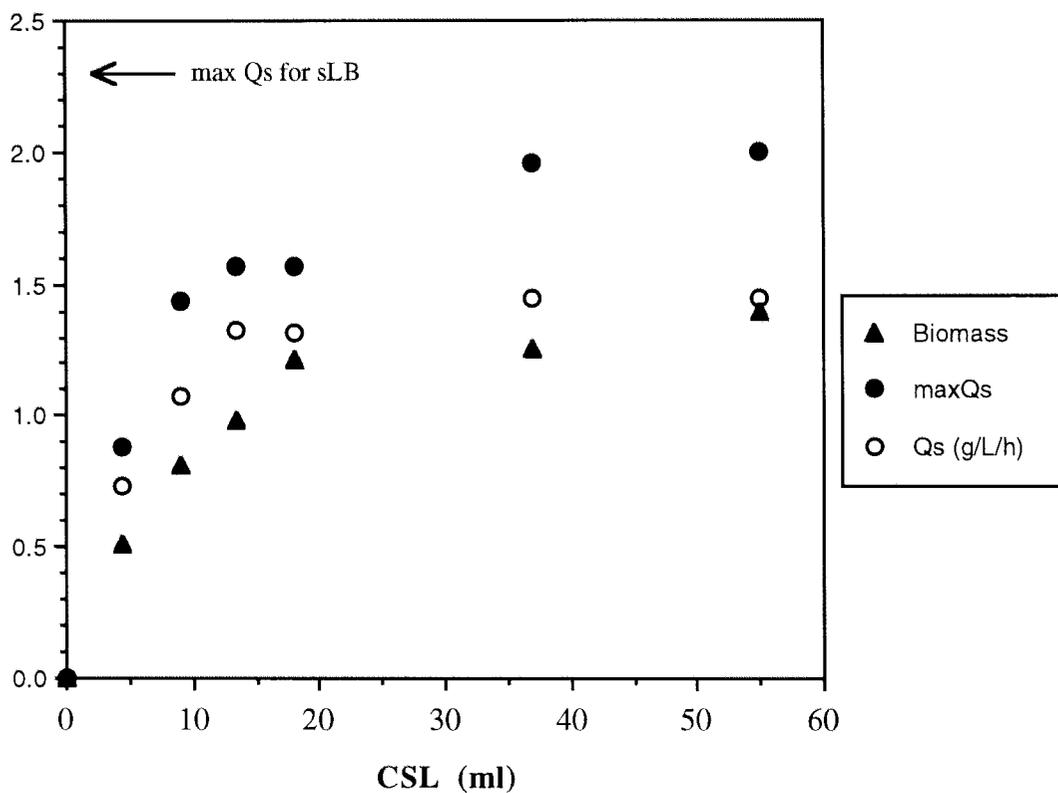


Figure 18 Cell concentration and the rate of xylose utilization as a function of CSL supplementation

Units: biomass (g dry wt. cells/L); Q_s and $maxQ_s$ (g Xylose/L.h)

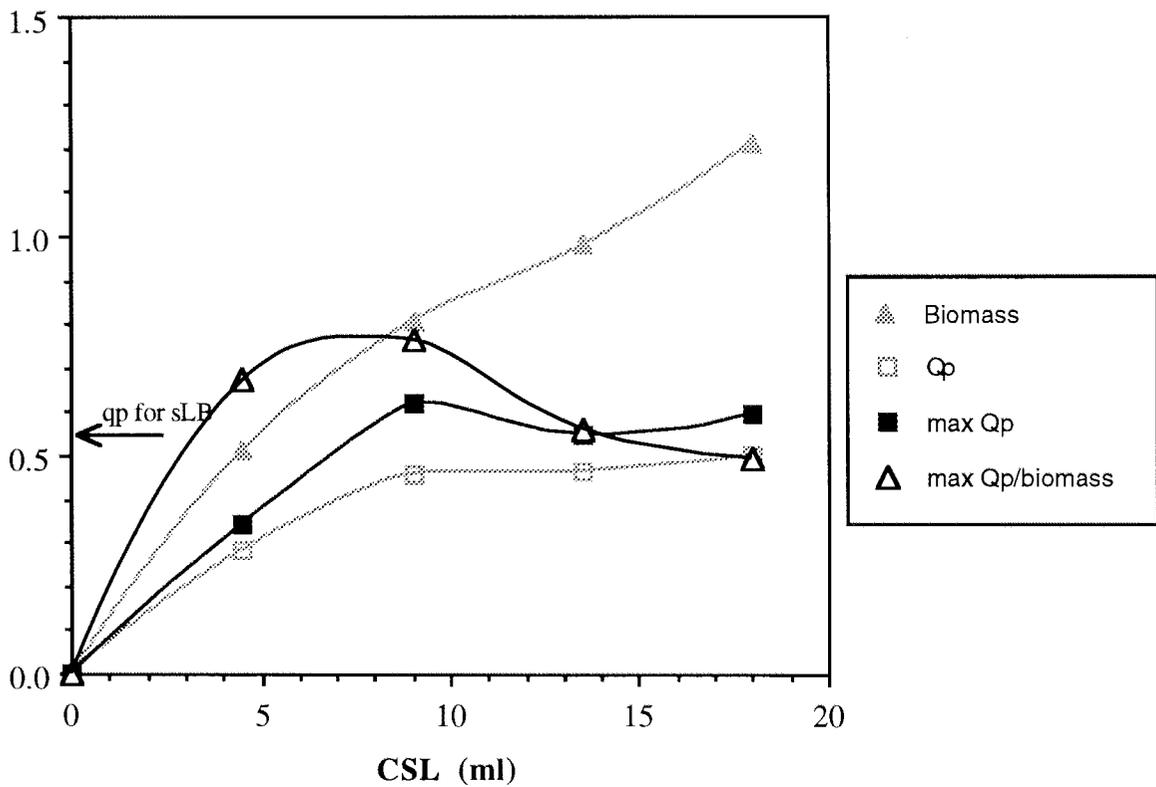
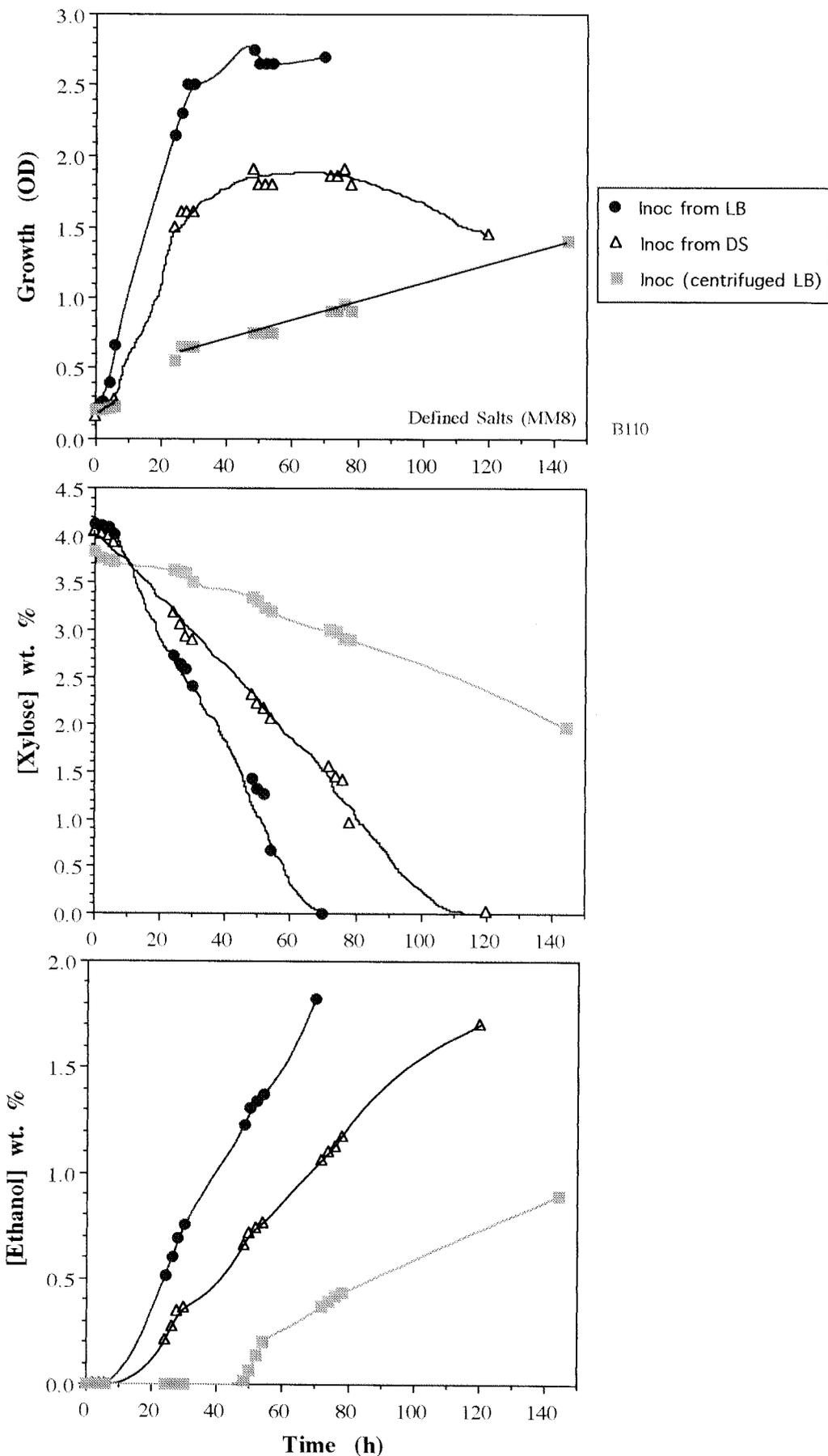


Figure 19 Cell concentration and ethanol productivity as a function of CSL supplementation

Units: biomass (g dry wt. cells/L); Q_p and $\max Q_p$ (g EtOH/L.h);
 Specific productivity = $\max Q_p/\text{biomass}$ (g EtOH/g cell.h)



B110

Figure 20 Growth and fermentation with a defined salts medium

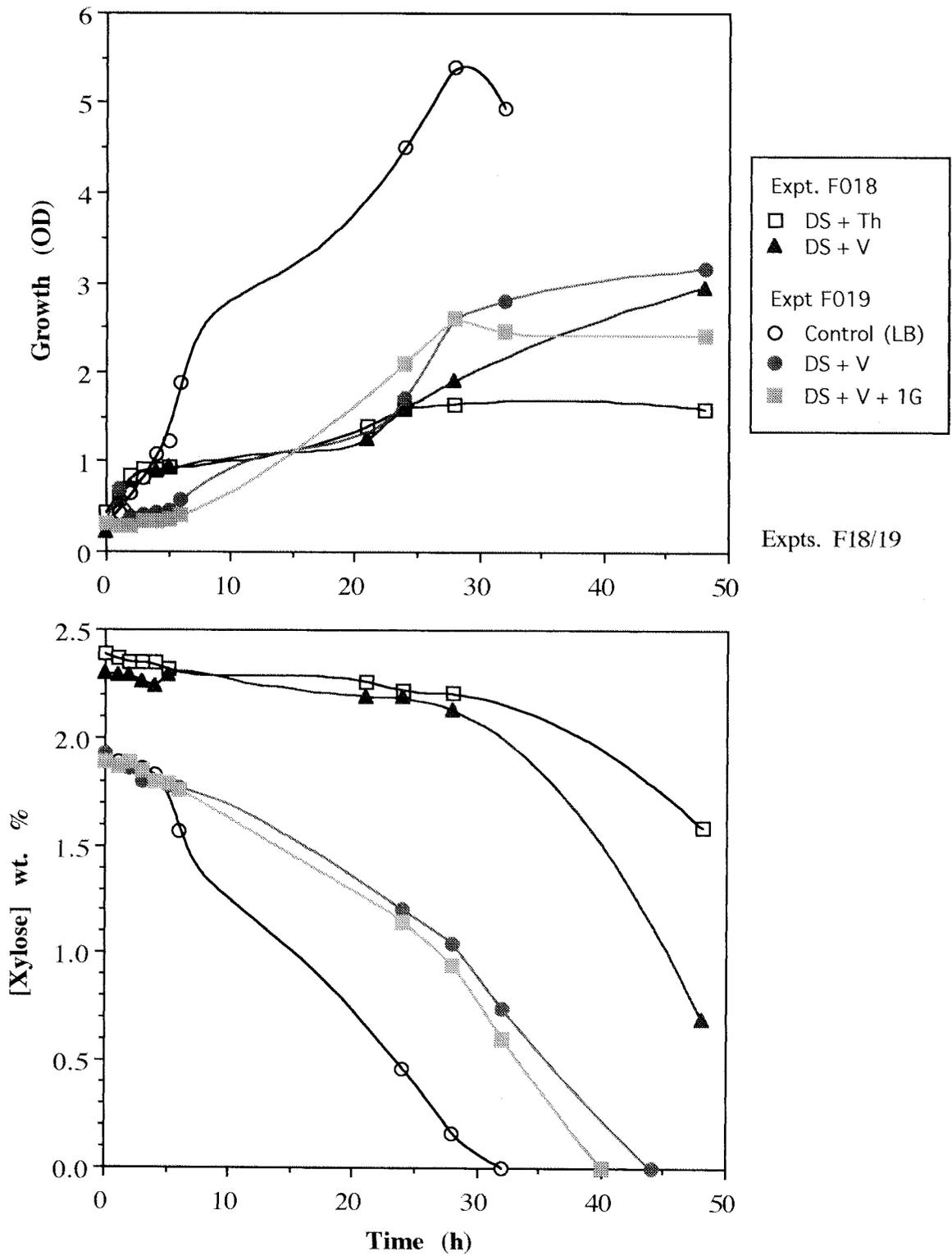


Figure 21 Flask fermentations - vitamin supplementation of defined salts medium

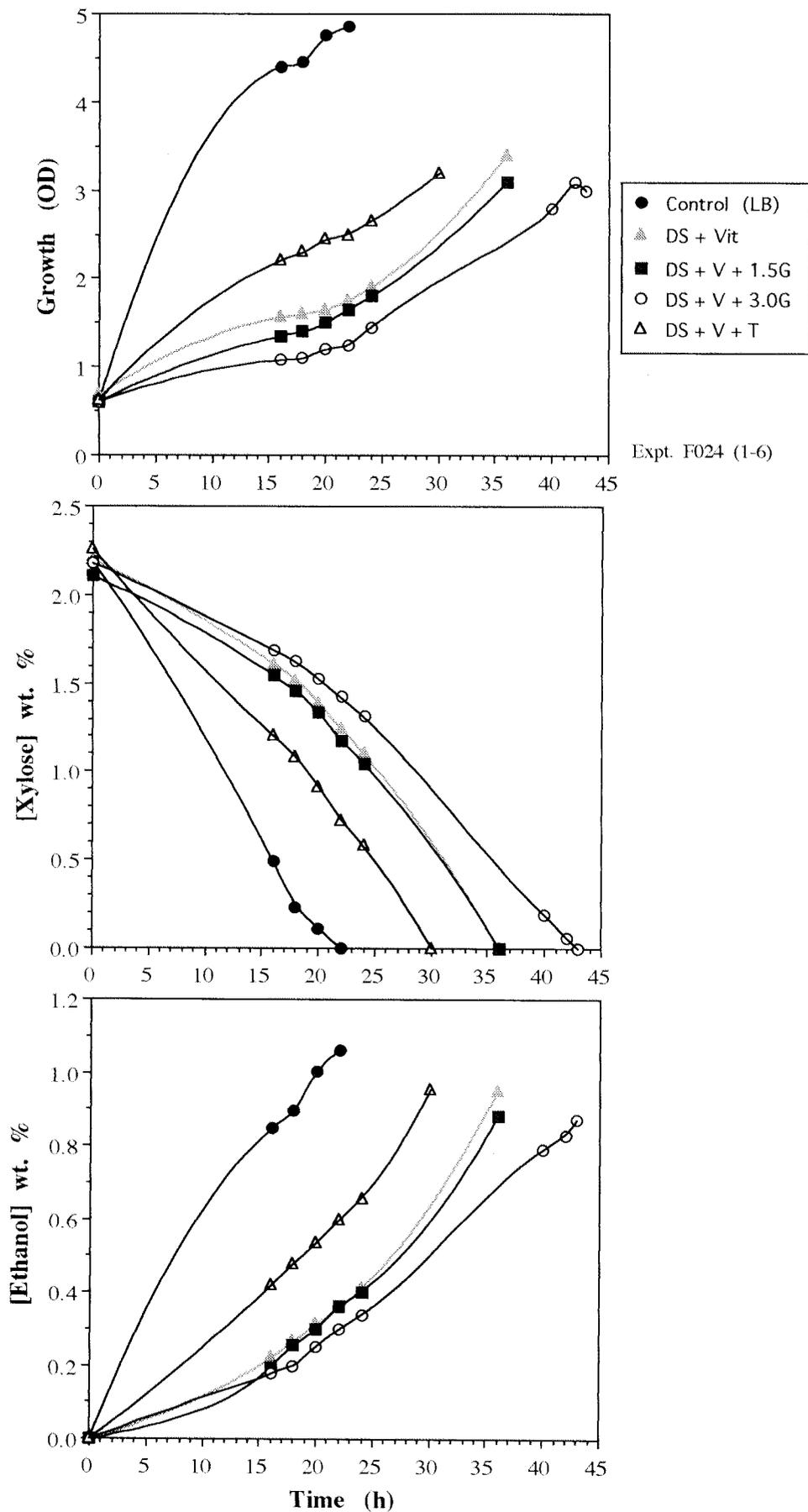


Figure 22 Flask fermentations - supplementation of DS medium

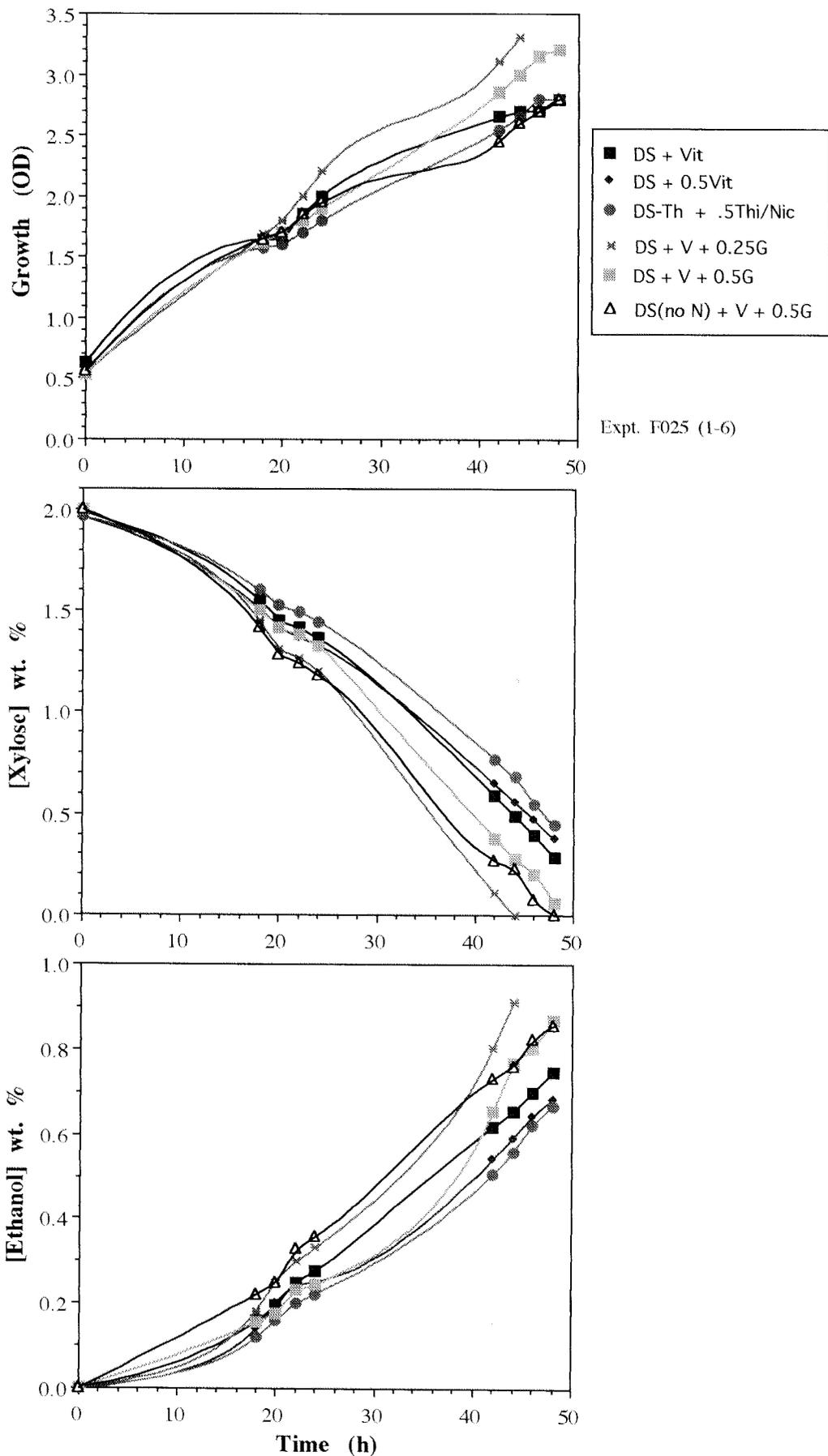


Figure 23 **Flask fermentations - supplementation of DS medium**

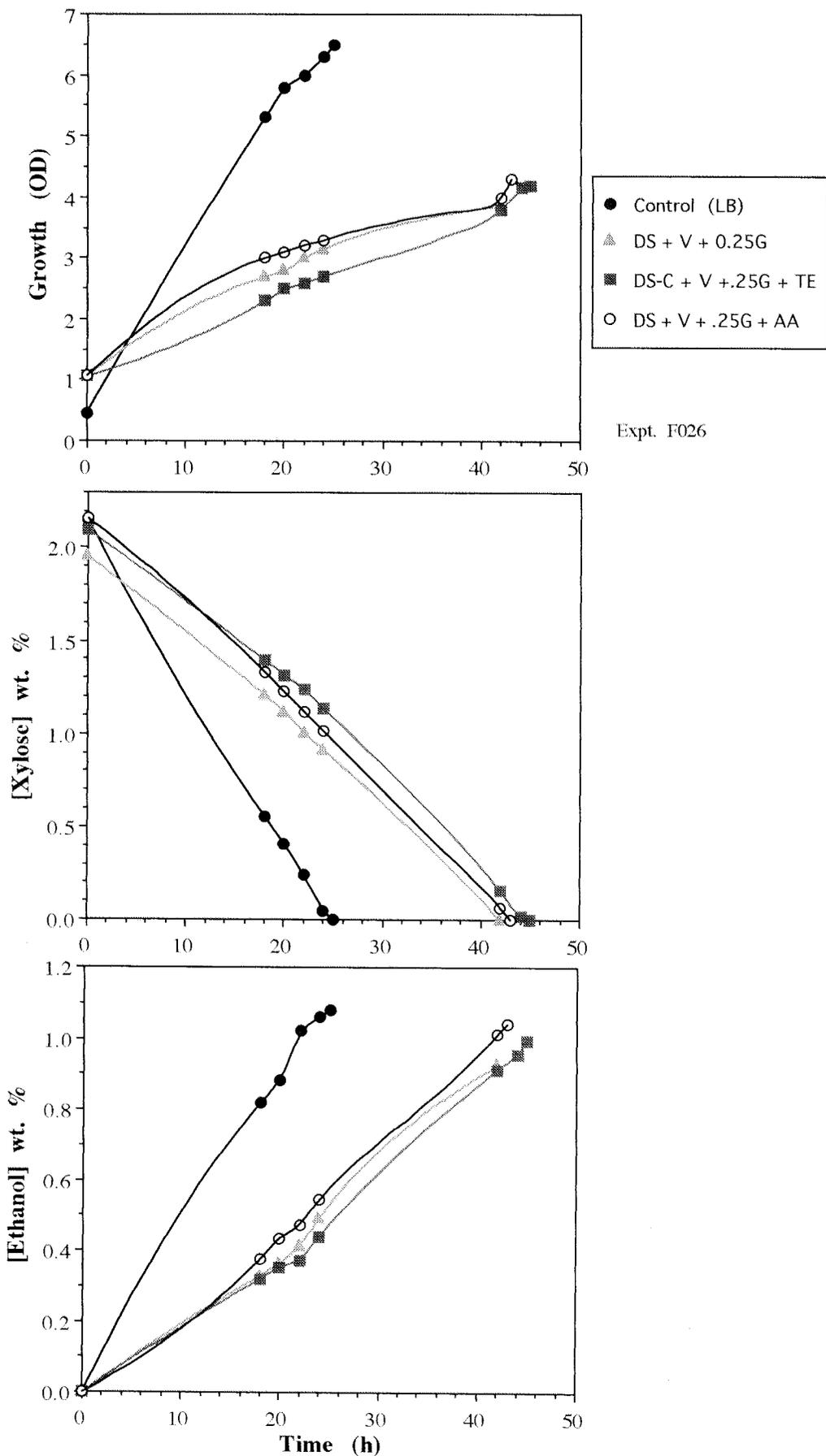


Figure 24 Flask fermentations - supplementation of DS medium

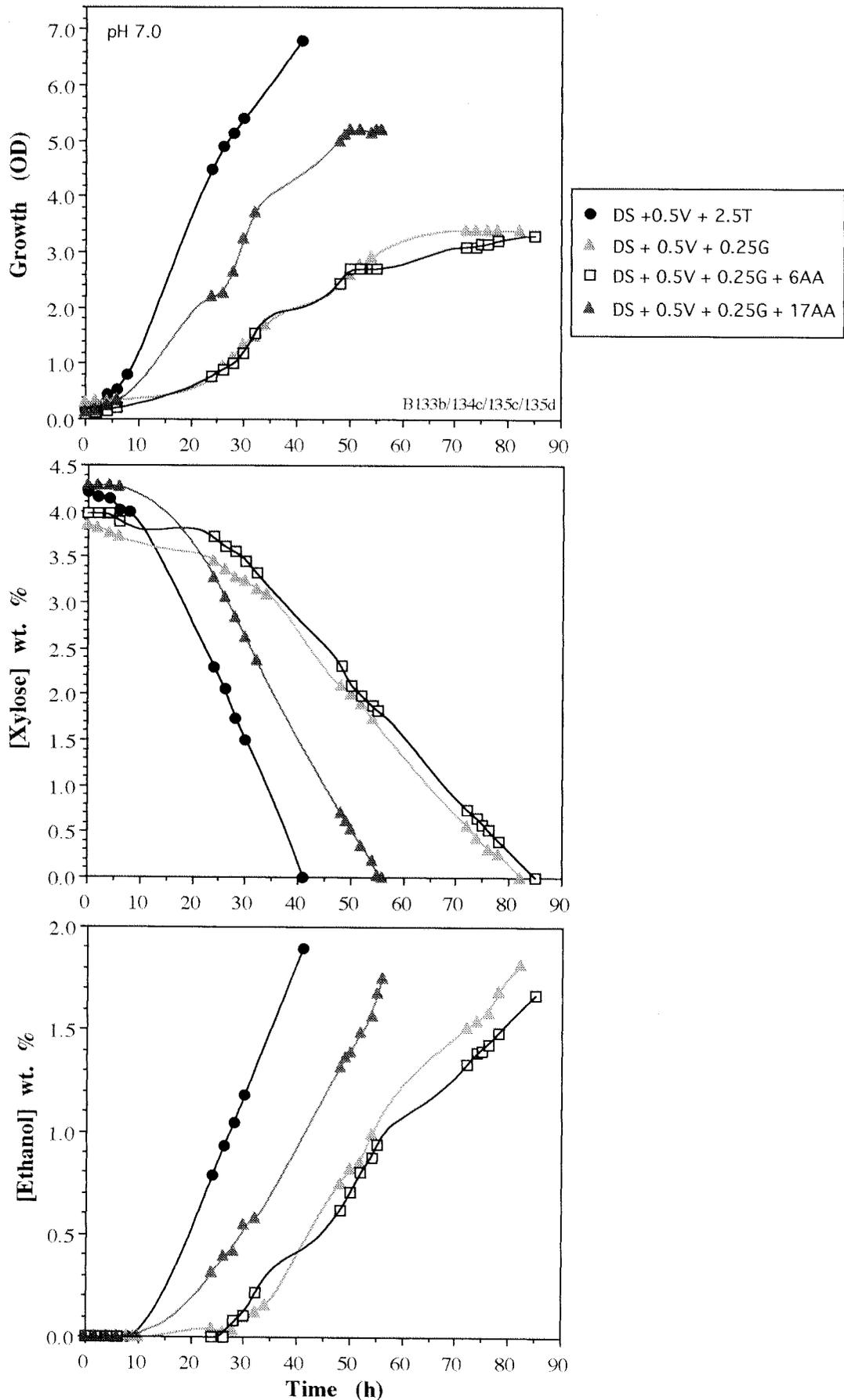


Figure 25 STR fermentations - supplementation of DS medium

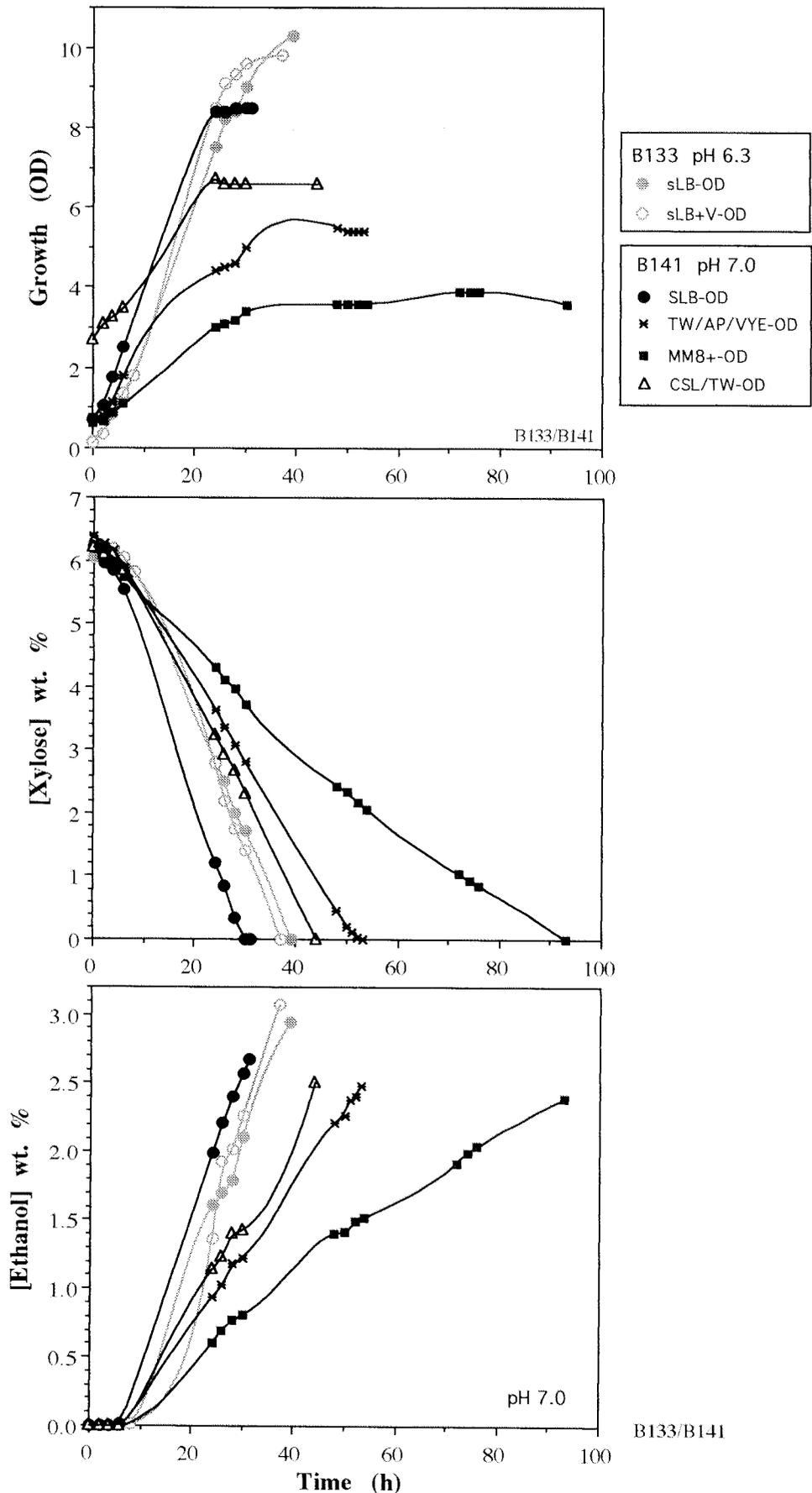


Figure 26 STR fermentations with selected media (6% xylose)

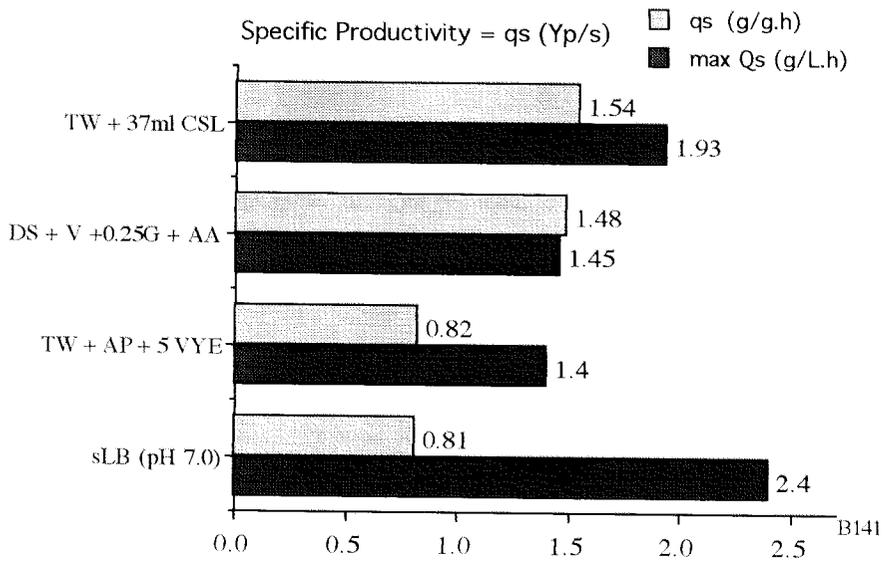
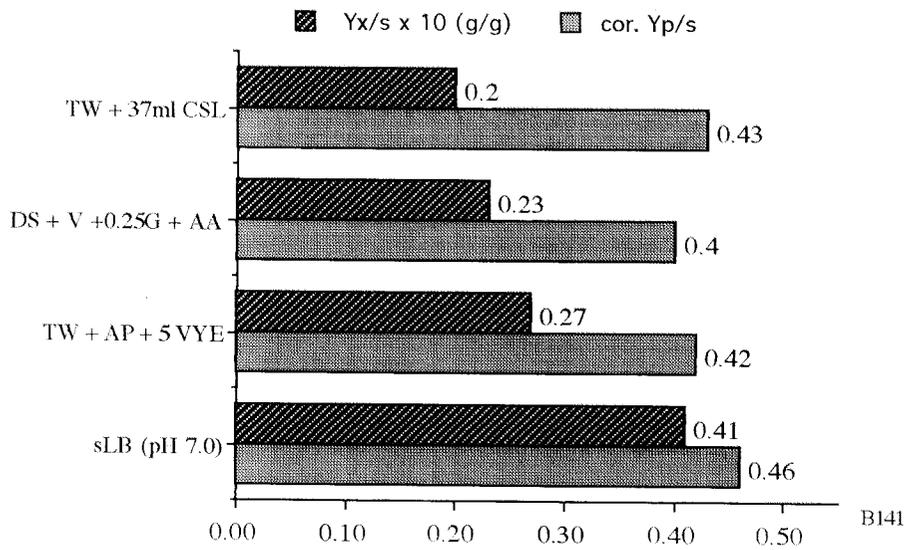
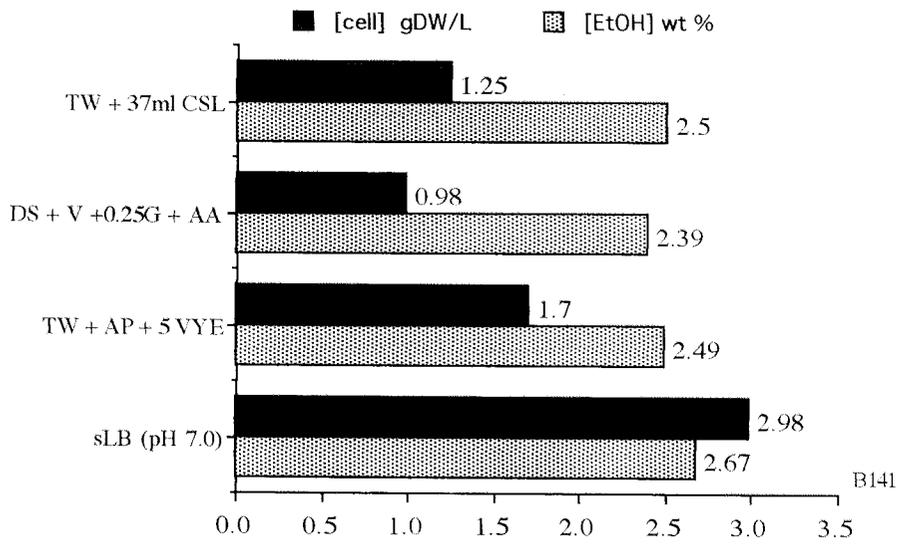


Figure 27 Comparative performance with selected media (6% xylose)

APPENDIX A

Compositional Information

Specifications for **Bacto Yeast Extract** and **Bacto Tryptone**
from Difco Laboratories Ltd. (Detroit, MI, USA)

Specifications for **Technical grade Yeast Extract** (Difco)

Specifications for **Veeprex B430** (yeast extract)
from Champlain Industries Ltd. (Mississauga, ON, Canada)

Specifications for **Pancase S** (casein hydrolysate)
from Champlain Industries Ltd. (Mississauga, ON, Canada)

DIFCO

LABORATORIES

BACTO® YEAST EXTRACT CODE: 0127

Bacto Yeast Extract is the water soluble portion of autolyzed fresh yeast. It is prepared and standardized especially for use in bacteriological culture media.

I. Identity Tests:

- A. Dehydrated Appearance: light beige, free-flowing, homogeneous.
- B. Solution: 1% solution, soluble in distilled or deionized water; light to medium amber, clear, may have a very slight precipitate. 2% solution, medium amber, clear, may have a very slight precipitate.

II. Assay:

- A. Loss on Drying (LOD): less than or equal to 4%.
- B. Reaction: the pH of a 1% solution after sterilization and cooling to 25°C should be 6.6 ± 0.2.
- C. Ash¹: less than or equal to 15%.
- D. Sodium Chloride¹: less than or equal to 5%.
- E. Nitrogen¹: 9 to 12%.
- F. Coagulable Protein¹: none.
- G. Cultural Response: A solution is prepared containing 1% Bacto Yeast Extract plus 0.5% sodium chloride and the pH is adjusted to 7.2 ± 0.2 using dilute NaOH. Tubes are inoculated with 100 1000 CFU's of test organisms & incubated at 35 ± 2°C for 18-48 hours.

TEST ORGANISMS

RECOVERY

<u>Neisseria meningitidis</u> ATCC® 13090	fair to good
* <u>Staphylococcus aureus</u> ATCC® 25923	good
<u>Streptococcus pneumoniae</u> ATCC® 6305	good

The above cultures are the minimum used for performance testing.

- * This culture is available as Bactrol™ Disks and are to be used as directed in Bactrol Disks Technical Information.

III. Stability: Product is stable to the expiry date on the label when stored below 30°C. Expiry date applies to product in its intact container.

¹ US Pharmacopeia XXII, 1990

09 SEPTEMBER 1993

DIFCO LABORATORIES • PO Box 331058 • DETROIT, MICHIGAN 48232-7058 USA • PHONE: 313 462-8500 • 800 521-0851
FAX: 313 462-8594 • FAX: 313 462-8517 (MARKETING) • TELEX: 23-5683 DIFCO LAB DET



Quality And Service Since 1895

<u>Percent</u>	<u>Neoptone</u>	<u>Protone</u>	<u>Casitone</u>	<u>Casamino Acids Technical</u>	<u>Casamino Acids</u>	<u>Yeast Extract</u>
Ash	3.90	2.50	6.66	30.8	3.64	10.1
Ether Soluble Extract	0.30	0.31				
Total N	14.33	15.41	13.00	7.85	11.15	9.18
Primary Proteose N	0.46	5.36				
Secondary Proteose N	3.03	7.60				
Neoptone N	10.72	2.40				
Ammonia N	0.12	0.05				
Free Amino N (Van Slyke)	2.82	1.86				
Imide N	1.23					
Orono-Amino N	7.56					
Di-Amino N	4.43					
Arginine	4.7	3.9	3.2	1.9	3.8	0.78
Aspartic Acid	6.7	10.8	6.5	4.0	0.49	5.1
Cystine (Sullivan)	0.39	0.27				
Glutamic Acid	15.2	8.1	20.0	12.6	5.1	6.5
Lysine	6.3	5.0	2.5	1.3	1.1	2.4
Histidine	2.3	5.9	2.1	1.4	2.3	0.94
Isoleucine	4.3	0.71	5.0	2.9	4.6	2.9
Leucine	8.4	13.6	8.2	4.0	9.9	3.6
Proline	6.4	10.3	7.0	4.4	6.7	4.0
Methionine	2.4	1.9	2.6	1.08	2.2	0.79
Phenylalanine	4.3	6.8	4.3	2.0	4.0	2.2
Threonine	3.7	4.6	4.2	2.2	3.9	3.4
Tryptophan	1.01	1.65	1.38	Nil	0.8	0.88
Tyrosine	5.3	3.0	2.8	0.52	1.9	0.60
Alanine	6.0	10.1	6.3	3.8	7.2	3.4
Organic Sulfur	0.63	0.45				
Inorganic Sulfur	0.09	0.16				
Phosphorus	0.112	0.15	0.22	0.29	0.35	0.29
Iron	0.0021	0.0099	0.0039	0.0101	0.0006	0.028
IO ₂	0.18	0.52	0.073	0.022	0.053	0.052
Potassium	0.85	0.06	0.12	0.16	0.88	0.042
Sodium	0.45	0.30	0.24	1.05	0.77	0.32
Magnesium	0.051	0.057	0.00060	0.0039	0.0032	0.030
Calcium	0.198	0.263	0.0913	0.0538	0.0025	0.040
Chlorine	0.84	0.38				
Chloride	0.84	0.38	0.425	21.34	11.2	0.190
<u>PM</u>						
Magnesium	5.8	6.0	3.7	5.7	7.6	7.2
Lead	5.00	9.00	5.00	3.00	4.00	16.00
Arsenic	0.37	0.46	0.32	0.00	0.50	0.11
Copper	19.00		10.00	8.00	10.00	19.00
Zinc	2.00	13.00	10.00	14.00	8.00	88.00
<u>Micrograms per Gram</u>						
Pyridoxine	5.0	0.24	1.1	0.025	0.073	20.0
Biotin	0.73	0.0021	0.34	0.050	0.102	1.4
Thiamine	3.4	0.17	0.48	0.02	0.12	3.2
Nicotinic Acid	134.00	2.1	24.00	2.5	2.7	279.00
Riboflavin	11.4	0.046	0.68	0.019	0.03	19.00

References:

Difco Laboratories, 1953 Difco Manual, Ninth Edition, Difco Laboratories, Detroit MI
 Schoenlein, H.W., 1957, Difco Laboratories, Personal Communication

DIFCO

LABORATORIES
P O BOX 331073
DETROIT MI 48232-7058 USA

PHONE 313 482-8500
TELEX 23-5683 DIFCO LAB DET
CABLE "DIFCO"
FAX 313 482-8594
FAX 313 482-8517 (Marketing)

YEAST EXTRACT TECHNICAL
CODE: 0886

Yeast Extract Technical is a low sodium, autolyzed yeast powder used as an ingredient in culture media when a standardized yeast extract is not essential.

I. Identity Tests:

- A. Powder: Light beige, homogeneous, free-flowing.
- B. Solution: 1% solution soluble in distilled or deionized water. Solution is light to medium amber in color, clear to very slightly opalescent, may have a precipitate.

II. Assay:

- A. Loss on Drying: Less than or equal to 4%
- B. Cultural Response: 2% solution with 0.5% Sodium Chloride is prepared and pH adjusted to 7.2 - 7.4 using dilute NaOH. Tubes are inoculated with approximately 100 - 1000 CFU's of the test organism and incubated at 35 ± 2°C for 18 - 24 hours.

<u>TEST ORGANISM</u>	<u>RECOVERY</u>
* <u>Escherichia coli</u> ATCC® 25922	Good
* <u>Streptococcus pyogenes</u> ATCC® 19615	Good

The above cultures are the minimum that should be used for test performance.

- * These cultures are available as Bactrol Disks and are to be used as directed in Bactrol Disk Technical Information.

III. Stability: The medium is stable to the expiry date on the label when stored below 30°C. Expiry date applies to medium in its intact container.

29 APRIL 1993

TYPICAL ANALYSIS OF DIFCO PEPTONES AND HYDROLYSATES

<u>Percent</u>	<u>Peptone</u>	<u>Proteose Peptone</u>	<u>Proteose Peptone No. 3</u>	<u>Tryptone</u>	<u>Tryptose</u>
Ash	3.53	9.61	4.90	7.28	8.44
Ether Soluble Extract	0.37	0.32		0.30	0.31
Total N	16.16	14.37	13.06	<u>13.14</u>	13.76
Primary Proteose N.	0.06	0.60		0.20	0.40
Secondary Proteose N	0.68	4.03		1.63	2.83
Peptone	15.38	9.74		11.29	10.52
Ammonia N	0.04	0.00		0.02	0.01
Free Amino N (Van Slyke)	3.20	2.66		4.73	3.70
Amide N	0.49	0.94		1.11	1.03
Mono-Amino N	9.42	7.61		7.31	7.46
Di-Amino N	4.07	4.51		3.45	3.98
Arginine	8.0	6.8	5.9	3.3	5.05
Aspartic Acid	5.9	7.4	6.6	6.4	6.9
Cystine (Sullivan)	0.22	0.56		0.19	0.38
Glutamic Acid	11.0	12.0	11.2	18.9	15.4
Glycine	23.0	11.6	8.9	2.4	7.0
Histidine	0.96	1.7	1.7	2.0	1.8
Isoleucine	2.0	3.3	3.3	4.8	4.0
Leucine	3.5	6.4	6.0	3.5	7.4
Lysine	4.3	5.3	5.1	6.8	6.0
Methionine	0.83	2.0	1.8	2.4	2.2
Phenylalanine	2.3	3.3	3.1	4.1	3.7
Threonine	1.6	3.5	3.2	3.1	3.3
Tryptophane	0.42	0.72	0.85	1.45	1.08
Tyrosine	2.3	3.4	0.36	7.1	5.2
Valine	3.2	4.4	4.0	6.3	5.3
Organic Sulfur	0.33	0.60		0.53	0.57
Inorganic Sulfur	0.29	0.04		0.04	0.04
Phosphorus	0.079	0.24	0.46	0.75	0.49
Iron	0.0023	0.0038	0.0044	0.0071	0.0054
AlO ₂	0.042	0.078	0.019	0.090	0.084
Potassium	0.22	0.70	0.21	0.30	0.50
Sodium	1.08	2.84	0.033	2.69	2.76
Magnesium	0.056	0.118	0.00048	0.045	0.081
Calcium	0.058	0.137	0.0396	0.096	0.116
Chlorine	0.27	3.95		0.29	2.77
Chloride	0.27	3.95	4.15	0.29	2.12
<u>PPM</u>					
Manganese	8.6	5.3	7.8	13.2	9.2
Lead	15.00	5.00	3.00	6.00	5.50
Arsenic	0.09	0.25	0.00	0.07	0.16
Copper	17.00	31.00	9.00	16.00	23.50
Zinc	18.00	44.00	37.00	30.00	37.00
<u>Micrograms Per Gram</u>					
Pyridoxine	2.5	3.0	4.1	2.6	2.8
Biotin	0.32	0.43	0.24	0.36	0.39
Thiamine	0.50	3.0	2.7	0.33	1.66
Nicotinic Acid	35.00	131.00	169.00	11.00	71.00
Riboflavin	4.00	11.00	13.00	0.18	5.59
Reaction, pH	7.0	6.8		7.2	7.3

pH 1 percent solution in distilled water after autoclaving 15 minutes at 121 C.

CHAMPLAIN Industries Limited

STANBRIDGE STATION QUEBEC CANADA J0J 2J0

TECHNICAL DATA

3A07

VEEPREX B430

DESCRIPTION

A Brewers Yeast Extract produced by natural enzyme hydrolysis especially processed to have a low sodium content.

VEEPREX B430 is a spray dried powder with a light tan colour which is completely soluble in water.

Suggested applications for this natural flavor enhancer include snacks, cheese biscuits and crackers, processed meats, bouillons, soups, cheese sauces, gravies.

TYPICAL ANALYSIS

Moisture	5.0%
Total Cl	1.6%
Total Na	0.4%
Total Nitrogen	8.5%
pH	5.6 ± 0.2
Colour	30 ± 5

MICROBIOLOGICAL SPECIFICATION

Standard Plate Count	10,000/g maximum
Salmonella	Neg. on 250 g
Staphylococcus	No coagulase positive
E-Coli	Neg.

INGREDIENTS DECLARATION

Yeast Extract, hydrogenated vegetable oil.

PACKAGING

3-ply Kraft paper bags with separate polyliner.
20 Kg. net, special packaging drums or boxes
available on request.

(Revised Feb/1984)

CHAMPLAIN Industries Limited

7200 WEST CHESTNUT AVENUE MISSISSAUGA ONTARIO CANADA L5N 5N1
 TELEPHONE: (905) 826-0801
 TELEFAX: (905) 826-0212
 TELEEX CG 218719 TELEFAX 416 826 0212 TELEPHONE 416 826 0801

NEW NUMBERS

**PANCASE™ S
 (Peptone)**

DESCRIPTION:

PANCASE™ S is a pancreatic digest of casein. This peptone is an excellent source of organic nitrogen in the form of amino acids, peptides, polypeptides and proteoses.

PANCASE™ S is a reproducible, uniform digest with superior growth-promoting qualities when used in microbiological culture media. The product has been processed to obtain high solubility for a wide variety of applications.

PANCASE™ S is also used as a growth nutrient in antibiotics production, toxins, enzymes, biologicals and various other products. It can serve as a basal medium for screening other nutrients in fermentation, and may be substituted in formulations requiring tryptic digest of casein. For nutritional applications, it is a well balanced source of amino acids* and can be used as a dietary supplement.

ANALYTICAL SPECIFICATIONS:

Moisture	:	5.0% (maximum)
Total Nitrogen	:	13.0 ± 1.0%
Amino Nitrogen/Total Nitrogen	:	40.0 ± 5.0%
Ash	:	6.0 ± 1.0%
pH (6% solution)	:	7.0 ± 0.2
Solubility (clear 30 deg. C)	:	250 g/litre

* Amino Acid profile is available on request.

MICROBIOLOGICAL SPECIFICATIONS:

Standard Plate Count	:	10,000/g maximum
Salmonella	:	negative on 250 g
Staphylococcus aureus (coagulase positive)	:	not detected
E. coli	:	not detected
Growth Properties	:	all test organisms show acceptable growth with peptone agar test

INGREDIENT STATEMENT: Casein Peptone

PACKAGING:

30 kgs net fibre drums with polyliners and epoxy coated steel covers.

APPENDIX B

Elemental Analysis of the following substances:

Pancase S (Champlain Ind. Ltd.)

Veeprex B430 (Champlain Ind. Ltd.)

Toronto tap water

Corn Steep Liquor (Nacan Products Ltd.)

Analyses were performed at the SLOWPOKE Reactor Facility
of the University of Toronto



SLOWPOKE Reactor Facility

University of Toronto, Toronto, Canada M5S 1A4

Tel(416)978-7129

To: Joyce Rousseau
 From: Ron Hancock
 1994 May 25

	Pancase S	Veeprex	Tap Water	(NH ₄) ₂ HPO ₄ mw = 132.06
<u>Al ppm*</u>	23±2.0023%	29±3.0029%	0.11±0.01	
Br ppm	≤3.4	≤3.6	0.036±0.006	
✓ Ca ppm	≤256.0256%	≤380.038%	40±2.004%	
✓ Cl ppm	400±40.04	21300±700	.0025.6±0.5	
✓ Cu ppm	≤26.0026%	≤23.0023%	≤0.089.0000089	
I ppm	≤2.0	≤2.0	≤0.010	
✗ K ppm	≤1700.17%	56000±3000	≤11.0011%	
✓ Mg ppm	≤752.0752%	≤690.069%	8.8±0.9.000887%	
✓ Mn ppm	≤0.576	≤0.56	≤0.0024	
✓ Na ppm	24400±700 2.44%	3700±100 .37%	12.5±0.3.00125	
✓ S ppm	≤4200.42	19000.9	≤46.0046	
Ti ppm	≤48	≤48	≤0.26	
V ppm	≤0.20	≤0.50	≤0.0012	
✓ Zn ppm	≤2900.29%	≤3000.3%	≤15.0015%	
N ₂ %	13%	8.5		
Note		.27	.27	

1. 10,000 ppm = 1%
2. Solid samples were analyzed 'as is', without drying.
- *. Some of the Al may be from a fast neutron reaction on P.

0.0070 0.0030/7
 mm mm

To: Joyce Rousseau
 From: Ron Hancock
 1994 July 13

Here are the data for the CSL sample that you wanted analyzed.

Element	ppm	
Ti	≤21	
I	≤1.2	
Br	≤2.4	
Mn	13±2	.0013% Mn
Mg	2600±200	.26% Mg
Cu	≤12	.12% Cu
Na	11000±300	1.10% Na
K	26000±1000	2.60% K
Al	27±1	0.27% Al
Cl	3100±100	.31% Cl
Ca	≤230	.023%
S	≤8000	0.8%

Note that a concentration of ≤xy ppm implies a 67% level of confidence that the concentration is less than xy ppm, and to a 95% level of confidence that the concentration is less than 2xy ppm.

APPENDIX C

Data Summary of Flask Fermentations

Summary of Comparative Parameters of *E. coli* ATCC 11303pLOI297 in Minimal Media at pH 7.

Flask #	Substrate Media Composition	SUBSTRATE USE		Q _S g S/L/h	Products		PRODUCTIVITY				Yield % C Recovery	Initial O.D.550
		[Xylose]~ g/l	mM		Biomass g/l (c)	*Y _x /s g X/g Xyl	[EtOH] g/L	Q _p g P/L/h	Y _p /s g P/g S			
F010	1 LB	17.05G	94.6	0.95	1.77	c1.37	.080	6.93	0.38	.41	95	0.10
	2 LB	21.83G	121.2	1.21	2.11	c1.71	.078	8.48	0.47	.39	88	0.10
	3 LB	21.10G	117.1	1.17	1.77	c1.37	.064	9.17	0.51	.43	96	0.10
F011	1 LB	9.60	64.0	0.48	1.02	c0.62	.064	5.35	0.27	.56	128	0.50
	2 LB	17.42	116.0	0.87	1.16	c0.76	.043	8.41	0.42	.48	108	0.50
	3 LB	17.70	118.0	0.88	0.95	c0.55	.031	8.80	0.44	.50	107	0.50
F012	1 LB	14.48	96.5	0.30	1.22	c0.82	.057	7.52	0.16	.52	115	0.26
	2 MM8-T/C+2.5DYE+5Tr	20.88	139.1	0.99	1.44	c1.14	.054	9.99	0.45	.48	116	0.26
	3 MM8-T/C+2.5DYE+5Tr	24.53	163.4	1.14	1.59	c1.29	.052	10.08	0.46	.41	99	0.26
	4 MM8-T/C+5DYE	23.93	159.4	1.18	1.92		.080	9.76	0.46	.41	97	0.26
	5 MM8-T/C+5DYE	26.96	179.6	1.32	1.86		.069	9.76	0.46	.36	86	0.26
	6 MM8-T/C+2.5DYE	24.76	165.0	1.20	1.47		.059	9.86	0.45	.36	93	0.26
	7 MM8-T/C+2.5DYE	26.30	175.2	1.01	1.74		.066	9.34	0.35	.36	85	0.26
	8 MM8	17.47	116.4	0.36	0.63		.036	5.77	0.12	.33	70	0.26
	9 MM8	16.49	109.9	0.34	0.69		.042	5.71	0.12	.35	75	0.26
F013	1 1/2 LB	19.74	131.5	0.99	1.67	c1.37	.069	9.60	0.48	.49	112	0.07
	2 1/2 LB+.125mM Mg	20.50	136.6	1.02	1.80	c1.50	.073	9.98	0.50	.49	108	0.08
	3 LB	18.99	126.5	0.90	1.80	c1.40	.074	10.30	0.45	.54	119	0.08
	4 LB +.5mM Mg	18.92	126.0	0.98	1.90	c1.50	.079	10.19	0.52	.54	122	0.13
	5 MM8-T/C+2.5DYE	17.40	115.9	0.87	1.32		.076	8.45	0.42	.48	110	0.15
	6 MM8-T/C+1.25DYE	15.79	105.2	0.79	1.17		.074	7.24	0.36	.46	106	0.09
	7 MM8-T/C+.625DYE	14.71	98.0	0.74	0.99		.067	6.48	0.32	.44	95	0.15
	8 MM8-T/C+2.5VYE	14.10	93.9	0.70	1.02		.072	6.17	0.31	.44	95	0.20
	9 MM8-T/C+.625VYE	7.60	50.6	0.38	0.78		.103	3.72	0.19	.49	109	0.12
F014	1 MM8-T/C+2.5DYE	20.64	137.5	0.97	1.50		.073	9.20	0.46	.45	106	0.345
	2 MM8-T/C+2.5DTYE	20.26	135.0	0.78	1.38		.068	9.25	0.43	.46	108	0.415
	3 MM8-T/C+2.5VYE	18.63	124.1	0.52	1.05		.056	8.19	0.25	.44	105	0.415
	4 MM8-T/C+2.5VBYE	20.58	137.1	0.53	1.20		.058	9.05	0.27	.44	103	0.45
	5 MM8-T/C+2.5GibYE	20.46	136.3	0.84	1.44		.070	9.57	0.34	.47	111	0.275
	6 MM8-T/C+2.5T-AYP	19.17	127.7	0.54	1.23		.064	8.34	0.27	.44	109	0.425

Substrate	SUBSTRATE USE	[Xylose]~		Products		PRODUCTIVITY		Yield		Initial O.D.550			
		g/l	mM	Q _S g S/L/h	Biomass g/l (c)	*Y _X /s g X/g Xyl	[EtOH] g/L	Q _p g P/L/h	Y _p /s g P/g S		% C Recovery		
F015	1	MM8-T/C+2.5DYE	20.00	133.2	0.94	1.48	.074	9.09	0.44	.46	106	0.30	
	2	MM8-T/C+2.5DYE(c-L)	20.00	133.2	0.90	1.26	.063	8.73	0.46	.44	100	0.30	
	3	MM8-T/C+2.5DYE(c-M)	20.00	133.2	1.04	1.23	.062	8.77	0.50	.44	100	0.16	
	4	MM8-T/C+2.5VYE	20.00	133.2	0.77	1.20	.060	8.72	0.29	.44	104	0.45	
	5	MM8-T/C+2.5VYE(f)	20.00	133.2	0.80	1.23	.062	9.34	0.34	.47	112	0.25	
	6	MM8-T/C+5.0VYE	20.00	133.2	0.84	1.26	.063	9.30	0.34	.47	107	0.45	
	7	MM8-T/C+2.5VYE+Tr	20.00	133.2	0.92	1.38	.069	9.36	0.41	.47	107	0.40	
	8	MM8-T/C+2.5VYE+P	20.00	133.2	0.90	1.32	.066	8.99	0.38	.45	104	0.35	
F016	1	MM8-T/C+2.5DYE	20.81	138.6	0.95	1.56	.075	10.25	0.46	.49	112	0.27	
	2	MM8-T/C+2.5VYE+Tr	24.38	162.4	1.17	1.62	.066	9.34	0.42	.38	90	0.32	
	3	TW+AC+.83Mg+2.5VYE+Ps	23.17	154.4	1.16	1.53	.066	9.85	0.49	.42	98	0.27	
	4	TW+AC+2.5VYE+Ps	21.32	142.0	1.09	1.56	.073	9.65	0.48	.45	104	0.23	
	5	TW+AP+2.5VYE+Ps	19.42	129.4	1.02	1.74	.090	10.03	0.55	.52	122	0.30	
	6	TW+2.5VYE+Ps	22.56	150.3	1.13	1.41	.062	9.29	0.46	.43	98	0.20	
F018	1	MM8-T	8.03	53.5	0.17	0.60	.075	2.12	0.04	.26	61	0.43	
	2	MM8+VS	16.14	107.5	0.33	0.90	.056	5.82	0.12	.36	77	0.22	
	3	MM8+VS+Tr	22.74	151.5	0.58	1.09	.048	10.71	0.27	.47	108	0.22	
	4	MM8+VS+CA	24.08	160.4	0.80	1.00	.042	9.88	0.33	.41	93	0.39	
	5	MM13(TW+18mlCSL)	0.00	0.0	-	0.12	-	0.00	-	-	-	1.20	
	6	MM13(TW+18mlCSL)	21.26	141.6	1.01	1.08	c0.96	.045	9.38	0.45	.44	98	1.14
	7	MM13+VS	22.03	146.8	1.05	1.12	c1.00	.045	9.44	0.45	.43	91	0.99
	8	MM13+VS+Tr	22.68	151.1	1.03	1.21	c1.09	.048	9.78	0.44	.43	106	1.11
	9	LB	0.00	0.0	-	0.40	-	0.04	-	-	-	0.50	
	10	LB	22.87	152.4	1.20	1.32	c0.92	.040	10.65	0.56	.47	105	0.39
F019	1	LB	19.00	126.6	0.59	1.64	c1.24	.065	9.07	0.28	.48	117	0.32
	2	MM8+VS	19.30	128.6	0.44	1.48		.077	7.81	0.18	.40	103	0.31
	3	MM8+VS+1G	18.91	126.0	0.47	1.25		.066	8.58	0.21	.45	112	0.31
	4	MM8+VS+1G/A	18.52	123.4	0.41	1.38		.074	8.52	0.19	.46	114	0.31
	5	MM8+VS+1G/A/C	18.63	124.1	0.39	1.18		.063	8.16	0.17	.44	110	0.31
	6	MM8+VS+1G/A/C/Ty/P	18.71	124.6	0.43	1.16		.062	8.97	0.20	.48	118	0.34
	7	MM8+VS+1G/A/C/Ty/P/H	18.50	123.2	0.40	0.92		.050	8.47	0.18	.46	110	0.32
	8	MM8+VS+G/A/C/Ty/P/H/S	18.30	121.9	0.40	0.90		.049	7.42	0.16	.40	91	0.35

<u>Substrate</u>	<u>SUBSTRATE USE</u>	<u>[Xylose]~</u>		<u>Products</u>		<u>PRODUCTIVITY</u>			<u>Yield</u>			
Flask #	Media Composition	g/l	mM	Q _S g S/L/h	Biomass g/l (c)	*Y _X /s g X/g Xyl	[EtOH] g/L	Q _p g P/L/h	Y _p /s g P/g S	% C Recovery	Initial O.D.550	
F020 1	LB	0.00	0.0	-	0.40	-	0.00	-	-	-	0.38	
2	LB	0.00	0.0	-	0.40	-	0.00	-	-	-	0.65	
3	LB	0.00	0.0	-	0.40	-	0.00	-	-	-	1.08	
4	LB	0.00	0.0	-	0.40	-	0.00	-	-	-	2.30	
5	TW+55mlCSL	0.00	0.0	-	0.27	-	0.11	-	-	-	3.40	
6	TW+55mlCSL	0.00	0.0	-	0.24	-	0.09	-	-	-	3.60	
7	TW+55mlCSL	0.00	0.0	-	0.30	-	0.14	-	-	-	3.90	
F021 1	LB(O)	0.00	0.0	-	0.40	-	0.02	-	-	-	0.50	
2	LB+.2% EtOH(O)	0.00	0.0	-	0.40	-	0.13	-	-	-	0.50	
3	LB(Cl)	0.00	0.0	-	0.40	-	0.01	-	-	-	0.48	
4	LB+.2% EtOH(Cl)	0.00	0.0	-	0.40	-	0.41	-	-	-	0.52	
5	LB(Cl)	0.00	0.0	-	0.40	-	0.02	-	-	-	0.47	
6	LB+.2% EtOH(Cl)	0.00	0.0	-	0.40	-	0.33	-	-	-	0.48	
7	LB(Cl)	0.00	0.0	-	0.40	-	0.01	-	-	-	0.48	
8	LB+.2% EtOH(Cl)	0.00	0.0	-	0.40	-	0.23	-	-	-	0.48	
F022 1	LB	22.32	148.7	0.86	1.64	c1.24	.056	9.75	0.38	.44	107	0.52
2	MM8-T(20mMN)	6.62	44.1	0.14	0.55		.083	2.84	0.06	.43	95	0.61
3	MM8-T(20mMN)+VS	11.33	75.5	0.24	0.74		.065	5.57	0.12	.49	105	0.60
4	MM8-T(10mMN)+1.5G	5.34	35.6	0.11	0.42		.079	2.14	0.04	.40	88	0.56
5	MM8-T(10mMN)+1.5G+VS	8.43	56.2	0.18	0.54		.064	4.04	0.08	.48	102	0.55
6	MM8-T(10mMN)+3G+VS	6.45	43.0	0.13	0.44		.068	2.28	0.05	.35	77	0.52
7	MM8-T(10mMN)+VS+1Tr	13.23	88.1	0.28	1.02		.077	0.26	0.01	.02	46	0.57
8	MM8-T(10mMN)+VS+2.5Tr	13.24	88.2	0.28	1.12		.084	0.26	0.01	.02	47	0.55
F023 1	mLB	0.00	0.0	-	0.26	-	0.51	-	-	-	0.49	
2	LB	0.00	0.0	-	0.40	-	0.68	-	-	-	0.51	
3	LB+.2% EtOH	0.00	0.0	-	0.40	-	0.40	-	-	-	0.50	
4	LB+.3% EtOH	0.00	0.0	-	0.40	-	0.46	-	-	-	0.45	
5	LB	16.40	109.3	0.68	1.56	c1.16	.071	8.31	0.35	.51	107	0.41
6	LB+.2% EtOH	16.84	112.2	0.70	1.55	c1.15	.068	8.65	0.36	.51	112	0.44
7	LB	31.54	210.1	1.31	2.14	c1.74	.055	16.40	0.68	.52	108	0.40
8	LB+.2% EtOH	31.53	210.1	1.31	2.19	c1.79	.057	16.07	0.67	.51	106	0.45

<u>Substrate</u>	<u>SUBSTRATE USE</u>	<u>[Xylose]~</u>		<u>Q_s</u>	<u>Biomass</u>		<u>*Y_{x/s}</u>	<u>[EtOH]</u>	<u>Q_p</u>	<u>Y_{p/s}</u>	<u>% C</u>	<u>Yield</u>
Flask #	Media Composition	g/l	mM	g S/L/h	g/l (c)	g X/g Xyl	g/L	g P/L/h	g P/g S	Recovery	Initial O.D.550	
F024 1	LB	21.88	145.8	0.99	1.40	c1.00	.046	10.61	0.48	.48	103	0.60
2	MM8+VS	21.97	146.4	0.61	0.83		.038	9.51	0.26	.43	97	0.68
3	MM8+VS+1.5G	21.06	140.3	0.58	0.83		.039	8.80	0.24	.42	94	0.60
4	MM8+VS+3G	21.80	145.2	0.51	0.76		.035	8.72	0.20	.40	90	0.60
5	MM8(0mMN)+VS+3G	22.25	148.2	0.47	0.80		.036	8.64	0.18	.39	93	0.60
6	MM8+VS+1Tr	22.56	150.3	0.75	0.84		.037	9.54	0.32	.42	96	0.62
7	MM8+VS+2.5Tr	21.69	144.5	0.90	1.08		.050	9.23	0.38	.42	97	0.62
F025 1	MM8+VS	16.96	113.0	0.35	0.84		.050	7.46	0.16	.44	109	0.63
2	MM8+1/2VS	15.81	105.3	0.33	0.84		.053	6.82	0.14	.43	106	0.58
3	MM8-T+1/2 Thi+1/2Nic	15.18	101.1	0.32	0.84		.055	6.68	0.14	.44	109	0.55
4	MM8+VS+.25G	19.64	130.8	0.45	0.99		.050	9.10	0.21	.46	108	0.56
5	MM8+VS+.5G	19.40	129.2	0.40	0.96		.049	8.67	0.18	.45	104	0.54
6	MM8(0mMN)+VS+.5G	19.95	132.9	0.42	0.84		.042	8.56	0.18	.43	105	0.56
F026 1	LB	21.53	143.4	0.86	1.88	c1.48	.069	10.81	0.43	.50	120	0.46
2	MM8+VS+.25G	19.55	130.2	0.46	1.13		.058	9.24	0.22	.47	106	1.06
3	MM8-C+VS+.25G+TE	20.98	139.8	0.47	1.27		.060	9.93	0.22	.47	105	1.06
4	MM8+VS+.25G+AA	21.60	143.9	0.50	1.24		.057	10.41	0.24	.48	108	1.06
5	MM8-C+VS+.25G+TE	20.56G	114.1	0.93	1.76		.086	9.33	0.42	.45	106	1.06
6	MM8+VS+.25G+AA	22.67G	125.8	1.26	1.90		.084	10.70	0.59	.47	109	1.06
7	LB	21.54G	119.6	1.80	2.28	c1.88	.087	10.98	0.92	.51	110	00.66

Notes:

~ = G indicates Glucose used as substrate

(c) = Corrected Biomass yield

* = Where Biomass is corrected for yield produced by media alone, this value is used in YX/S

Codes

LB	= Luria Broth	MM8	= Minimal Media #8
-T/C	= - Thiamine & citric acid	DYE	= Difco Yeast Extract(g/L)
Tr	= Tryptone(g/L)	Mg	= Magnesium sulphate
VYE	= Veeplex B430 Yeast Extract(g/L)	VBYE	= Veeplex B800 Yeast Extract(g/L)
Gib YE	= Gibco Yeast Extract(g/L)	T-AYP	= Tureen AYP-65(g/L)
(c-L)	= Inoculum centrifuged & resuspended in LB	(c-M)	= Inoculum centrifuged & resuspended in MM8
(f)	= Media filter sterilized	CA	= 1 g/L Difco Casamino acids
TW	= Toronto Tap Water	CSL	= Corn Steep Liquor(NACAN Products Ltd)(ml/L)
MM13	= Toronto Tap Water & 18 ml Corn Steep Liquor	VS	= Vitamin Stock added at 10 ml/L
G	= Glutamic acid(g/L)	A	= 0.3 g/L Aspartic acid
C	= 50 mg/L Cysteine	Ty	= 50 mg/L Tyrosine
P	= 50 mg/L Phenylalanine	H	= 50 mg/L Histidine
S	= 50 mg/L Serine	(C)(O)	= Flask opened & sampled or kept closed for 24 hr
TE	= 5.0 g/L Disodium EDTA + 0.22 g/L Zinc Sulphate.7H ₂ O + 0.50 g/L Ferrous Sulphate		
AA	= Amino acid cocktail = 50 mg/L each Aspartic acid,Tyrosine,Tryptophan,Phenylalanine & Histidine		

APPENDIX D

Data Summary of pH-stat STR Batch Fermentations

**Product Distribution by *E. coli* ATCC 11303 with plasmid LOI297
Anaerobic Growth**

^MP strain

*Mixture of two colony types

rResidual carbon

#Inoculum centrifuged at 10,000 RPM for 10 min.

mInoculum grown in MM

vVitamins added

<u>Substrate</u>	<u>SUBSTRATE USE</u>		<u>Products</u>				<u>PRODUCTIVITY</u>				<u>Yield</u>				
Batch #	[Xyl] g/l	mM	Q _S g S/L/h	Max.Q _S g S/L/h	Biom. g/l	[EtOH] mM	Succinic mM	Lactic mM	Acetic mM	Formic mM	Q _p g P/L/h	Max.Q _p g P/L/h	Y _{p/s} g P/g Xyl	pH	% C Recov.
Medium Composition: Luria Broth															
B104a	44.6	297.1	1.72	2.26	2.34	458.4	31.9	0.0	0.4	0.0	0.81	1.39	.47	6.3	108
B104b	45.1	300.3	1.80	2.24	2.31	411.3	29.3	0.0	1.3	0.0	0.76	1.91	.42	7.0	96
Medium Composition: Luria Broth + 0.5 mM Mg															
B101a^	21.9	145.9	0.88	1.10	1.95	77.7	0.4	37.1	63.8	0.0	0.04	0.58	.16	7.0	84
B101b^	42.7	284.4	1.24	1.57	2.28	5.6	.8	1.7	28.2	0.0	0.01	0.01	.08	7.0	21
B101c^	63.4	422.4	1.47	2.62	4.18	68.6	0.0	241.0	71.6	0.0	0.07	1.37	.05	7.0	62
B104c	48.1	320.1	2.00	2.62	2.60	429.1	23.8	0.0	9.6	0.0	0.82	1.09	.41	7.0	95
Medium Composition: Luria Broth + 0.5 mM Mg + 17 mM P															
B105a	0.0	0.0	-	-	0.42	0.4	0.0	0.0	0.0	0.0	-	-	-	7.0	-
B105b	39.3	261.9	1.31	1.95	2.06	411.3	33.9	0.0	22.4	0.0	0.63	1.02	.48	7.0	116
B106a	20.5	136.6	1.46	2.21	1.40	205.3	14.4	0.0	9.0	0.0	0.68	1.17	.46	7.0	111
B106b	43.1	287.4	1.72	2.02	2.07	428.7	27.9	0.0	10.6	0.0	0.79	2.47	.46	7.0	105
B106c	57.0	380.0	2.19	5.31	2.82	554.4	43.4	0.0	14.4	0.0	0.98	2.78	.45	7.0	105
B111a	42.4	282.8	1.93	2.29	2.28	445.4	31.2	0.0	13.2	0.0	0.93	1.25	.48	7.0	113
B119a	41.5	276.7	1.48	1.78	1.87	465.8	15.6	0.0	1.3	0.0	0.77	1.11	.52	6.3	111
B133c	60.3	401.8	1.55	1.90	2.74	637.9	24.2	22.0	10.2	0.0	0.75	0.99	.49	6.3	110
B133dv	62.7	417.5	1.69	2.28	2.71	667.9	24.9	8.2	0.8	0.0	0.83	0.85	.49	6.3	107
B141a	62.7	417.5	2.02	2.40	2.98	580.6	25.9	0.0	14.5	0.0	0.86	0.98	.43	7.0	96
B142a	45.1	300.4	1.07	1.65	1.74	479.0	15.6	0.0	0.0	0.0	0.52	0.82	.49	6.0	104
B142b	43.6	290.7	1.15	1.83	1.78	432.4	21.8	0.0	9.4	0.0	0.52	0.86	.46	6.3	102
B142c	40.8	271.5	1.23	2.02	1.84	397.9	19.9	0.0	11.5	0.0	0.56	0.79	.45	6.6	102
B142d	42.6	283.9	1.18	1.64	2.01	403.5	15.5	0.0	10.5	0.0	0.52	0.66	.44	7.0	96

**Product Distribution by *E. coli* ATCC 11303 with plasmid LOI297
Anaerobic Growth**

Substrate	SUBSTRATE USE						Products				PRODUCTIVITY Yield					
	Batch #	[Xyl]		Q _S	Max.Q _S	Biom.	[EtOH]	Succinic	Lactic	Acetic	Formic	Q _p	Max.Q _p	Y _{p/s}	pH	% C
	g/l	mM	g S/L/h	g S/L/h	g/l	mM	mM	mM	mM	mM	g P/L/h	g P/L/h	g P/g Xyl		Recov.	
Medium Composition: Luria Broth + 0.83 mM Mg																
B103a	25.4	169.0	0.84	1.04	1.50	207.7	24.8	0.0	3.5	0.0	0.32	0.81	.38	7.0	94	
B103b	44.4	295.5	1.71	2.28	2.27	390.7	2.8	0.0	2.8	0.0	0.69	0.92	.40	7.0	87	
B103c	61.5	409.8	2.37	3.08	2.94	533.5	35.9	0.0	69.3	0.0	0.94	3.00	.40	7.0	94	
Medium Composition: mLuria Broth																
B133a	42.1	280.7	1.28	1.96	1.88	435.4	20.2	0.1	0.8	0.0	0.61	0.78	.48	7.0	104	
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)																
B102a*	21.0	140.1	0.68	1.24	0.72	127.0	12.4	0.0	6.9	0.0	0.19	0.32	.28	7.0	69	
B102b*	41.1	273.5	0.68	0.82	0.91	282.4	27.4	0.0	15.2	0.0	0.22	0.36	.32	7.0	76	
B102c*	60.1	400.3	0.57	0.78	0.94	416.5	28.2	0.0	14.1	0.0	0.18	0.24	.32	7.0	72	
B107am	20.5	136.4	0.20	0.24	0.52	176.2	12.2	0.0	9.7	0.0	0.08	0.10	.40	7.0	95	
B107bmr	45.2	301.2	0.21	0.24	0.58	250.9	0.0	0.0	10.4	0.0	0.08	0.18	.39	7.0	81	
B107cmr	61.6	410.6	0.22	0.32	0.75	269.4	0.0	0.0	14.2	0.0	0.09	0.18	.38	7.0	82	
B110am	40.5	270.0	0.34	0.36	0.44	369.0	15.9	0.0	18.0	0.0	0.14	0.18	.42	7.0	92	
B110b	41.2	274.4	0.59	0.68	0.65	396.6	26.5	0.0	15.8	0.0	0.26	0.28	.44	7.0	100	
B110c#r	38.4	255.8	0.13	0.24	0.27	193.0	0.0	0.0	10.8	0.0	0.06	0.30	.48	7.0	100	
B110d#r	39.4	262.3	0.24	0.29	0.29	296.1	13.5	0.0	12.5	0.0	0.09	0.28	.44	7.0	86	
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)+2.5 g/L Difco YE + 5 g/L Tryptone																
B111b	41.8	278.7	1.74	2.00	2.08	451.9	24.4	0.0	8.7	0.0	0.87	1.15	.50	7.0	112	
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)+2.5 g/L Difco YE																
B111c	49.3	328.7	1.50	1.80	2.06	475.8	31.2	0.0	11.4	0.0	0.66	0.75	.44	7.0	101	
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)+0.625 g/L Difco YE																
B113a	47.4	315.6	1.18	1.52	1.20	426.5	30.1	0.0	11.2	0.0	0.49	0.70	.42	7.0	94	
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)+2.5 g/L Veeplex B430 YE																
B111d	50.0	333.1	0.89	1.01	1.20	478.2	30.4	0.0	14.9	0.0	0.39	0.52	.44	7.0	99	
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P) + 2.5 g/L Veeplex B430 YE + 1.0 g/L Tryptone																
B113b	21.5	143.2	0.83	1.54	1.33	189.9	13.2	0.0	0.7	0.0	0.34	0.58	.41	7.0	95	
Medium Composition: LR MM 8 (0.83mM Mg + 20 mM P) + 2.5 g/L Veeplex B430 YE + 1.0 g/L Pancase S																
B113c	48.7	324.4	1.43	1.83	1.42	451.2	29.6	0.0	9.0	0.0	0.62	1.00	.44	7.0	96	

Product Distribution by *E. coli* ATCC 11303 with plasmid LOI297SubstrateSUBSTRATE USEProductsPRODUCTIVITY Yield

Batch #	[Xyl] g/l mM	Q _S g S/L/h	Max.Q _S g S/L/h	Biom. g/l	[EtOH] mM	Succinic mM	Lactic mM	Acetic mM	Formic mM	Q _p g P/L/h	Max.Q _p g P/L/h	Y _{p/s} g P/g Xyl	pH	% C Recov.
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P) + 5.0 g/L Veeprax B430 YE														
B113d	48.6 323.4	1.35	1.98	1.38	453.9	33.1	0.0	13.0	0.0	0.58	0.86	.43	7.0	98
Medium Composition: tap H ₂ O + (NH ₄) ₂ HPO ₄ +2.5 g/L Veeprax B430 YE														
B114a	39.9 265.7	1.33	1.63	1.45	399.6	33.7	0.0	14.4	0.0	0.61	0.85	.46	7.0	108
Medium Composition: tap H ₂ O + (NH ₄) ₂ HPO ₄ +5.0 g/L Veeprax B430 YE														
B141b	63.9 426.0	1.21	1.40	1.70	537.2	51.4	0.0	24.6	0.0	0.47	0.52	.39	7.0	92
Medium Composition: tap H ₂ O + (NH ₄) ₂ HPO ₄ +1.0 g/L Pancase S														
B114b	41.7 278.1	1.30	1.64	1.39	390.7	33.4	0.0	14.2	0.0	0.56	0.72	.44	7.0	101
Medium Composition: MM 11														
B114c	40.4 269.2	1.44	1.66	1.58	376.6	38.9	0.0	15.9	0.0	0.62	0.68	.43	7.0	104
Medium Composition: MM 12														
B115a	41.7 278.1	1.35	1.74	1.32	412.0	34.5	0.0	12.4	0.0	0.61	0.80	.45	7.0	105
B117a	40.0 266.4	1.33	1.69	1.46	401.3	33.2	0.0	0.0	0.0	0.62	0.69	.46	6.3	105
B117b	38.1 254.3	1.19	1.52	1.40	378.1	30.8	0.0	10.7	0.0	0.58	0.67	.46	7.0	106
B118a	43.7 291.3	1.51	1.91	1.40	397.2	36.7	0.0	5.2	0.0	0.63	0.95	.42	7.0	96
B118b	42.1 280.4	1.40	1.89	1.27	394.2	41.4	0.0	10.5	0.0	0.60	0.85	.43	7.0	102
B118c	42.1 280.7	1.28	1.36	1.33	404.4	37.6	0.0	0.4	0.0	0.56	0.81	.44	6.3	101
B120a	0.4 2.7	0.10	0.33	0.32	0.4	0.04	0.0	0.0	0.0	0.01	0.02	.05	6.3	106
Medium Composition: CSL & tap water (mL/L CSL, 1=55, 2=37, 3=18, 4=13.5, 5=9, 6=4.5)														
B121a-1	36.3 241.7	1.45	2.00	1.40	438.9	28.4	0.0	18.1	0.0	0.81	1.10	.56	7.0	128
B121b-2	36.3 241.7	1.45	1.96	1.26	424.6	25.3	0.0	14.3	0.0	0.78	1.10	.54	7.0	122
B141d-2	62.2 414.1	1.41	1.93	1.25	543.5	50.0	0.0	10.2	0.0	0.60	0.69	.40	7.0	92
B121c-3	35.9 238.9	1.33	1.64	1.10	387.0	46.8	0.0	16.6	0.0	0.66	0.85	.50	7.0	105
B124a-3	44.7 297.9	1.32	1.57	1.21	398.1	49.6	0.0	16.8	0.0	0.54	0.63	.41	7.0	100
B124b-4	46.5 310.0	1.33	1.57	0.98	376.8	45.7	0.0	15.8	0.0	0.50	0.50	.37	7.0	90
B124c-5	45.0 300.1	1.07	1.44	0.81	441.7	51.1	0.0	27.3	0.0	0.48	0.67	.45	7.0	110
B124d-6	45.2 301.1	0.73	0.88	0.51	411.5	48.3	0.0	25.0	0.0	0.30	0.36	.42	7.0	100
B125a-3	77.0 512.7	1.18	2.20	1.84	693.9	86.0	0.0	28.6	0.0	0.49	0.71	.42	7.0	101
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)+2.5 g/L Difco Tryptone + Vitamins														
B133b	42.2 280.9	1.03	1.35	1.82	411.8	22.9	3.5	11.9	0.0	0.46	0.65	.45	6.3	103

Product Distribution by *E. coli* ATCC 11303 with plasmid LOI297

Substrate **SUBSTRATE USE** **Products** **PRODUCTIVITY** **Yield**

Batch #	[Xyl] g/l mM	Q _S g S/L/h	Max.Q _S g S/L/h	Biom. g/l	[EtOH] mM	Succinic mM	Lactic mM	Acetic mM	Formic mM	Q _p g P/L/h	Max.Q _p g P/L/h	Y _{p/s} g P/g Xyl	pH	% C Recov.
Medium Composition: LR MM 8 (0.83 mM Mg + 20 mM P)+1/2 Vitamins +.25 g/L Glutamic acid(A=Amino acids)														
B134c	38.6 256.8	0.47	0.66	1.05	393.3	19.6	0.0	17.9	0.0	0.22	0.39	.47	7.0	106
B135c6A	39.82 265.3	0.47	0.63	0.98	362.9	18.8	0.0	11.5	0.0	0.20	0.26	.42	7.0	93
B135d17A	42.87 285.6	0.76	1.05	1.59	380.7	15.5	14.4	10.3	0.0	0.31	0.44	.41	7.0	94
B141cV17A	62.27 414.8	0.67	1.45	0.98	519.2	36.8	0.0	25.0	0.0	0.26	0.36	.38	7.0	88