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(*Ph.D. Thesis)

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Nutritional and Environmental Factors in Ethanol Fermentation by Saccharomyces cerevisiae

by

Harry Wong

Abstract

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Using Saccharomyces cerevisiae as a model system, a basic study of the nutritional and environmental factors in ethanol fermentation was carried out to provide fundamental and practical bases for design of fermentation media and culture conditions. The requirements for all active medium components need to be determined in order to establish balanced media, which are important to reduce raw materials costs and to minimize inhibition from buildup of excess feed components in recycle processes with selective ethanol removal.

Pulse injection of nutrients into continuous cultures was an effective method for screening active nutrients. In a systematic sensitivity analysis the effect of feed concentration of these individual nutrients was then determined and allowed formulation of media optimal with respect to the major fermentation parameters. Biotin, pantothenate, myo-inositol, potassium and phosphates appeared to stimulate growth preferentially to ethanol production. In contrast, thiamine and pyridoxine appeared to enhance specific ethanol productivity. The effect of ammonium sulfate depended on concentration. A conceptual model was proposed to relate the effects of these nutrients to biochemical pathways and functions. With these data and model the minimum cost combination of raw materials to achieve a medium of well defined components can be determined with a linear program. This computer program shows that many growth factors and minerals can be added to media more economically as pure components than as fractions of complex factors.

The effect of dissolved oxygen was studied from essentially zero to 346 mmHg oxygen tension, showing a continuous decline in specific ethanol productivity with increasing oxygen over this range. Oxygen uptake rates under fermentative conditions showed approximately saturation kinetics as a function of dissolved oxygen tension for varying dilution rates. This fermentative oxygen demand of order 30 mg O_2 /g dry cells was related to requirements for biosynthesis of unsaturated fatty acids and ergosterol. The internal cell reaction rate for O_2 appeared to control the rate of O_2 uptake, which appeared to be by passive diffusion.

Long term continuous cultures resulted in decreased media requirements for growth factors and increased tolerance for ethanol inhibition, most probably through adaptation. An ethanol productivity of 5.6 g/L-hr in continuous culture was achieved with a completely synthetic medium with the improved culture. Specific media requirements for a process need to be determined with the actual organism, raw materials and environmental conditions to be used. Many of the trends observed in this study should, nevertheless, be applicable to organisms with similar metabolism.

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Chapter 1

1. Introduction

1.1 Historical Development and Usages of Ethanol

Alcoholic fermentations predate the earliest known civilizations. The ancient Greeks attributed the invention of wine from grapes and other fruit juices to the god Dionysus. Beer making was reported by about 2500 B.C. by the Egyptians from barley, by the Chinese from rice, and later by American Indians from corn. The Arabs, Chinese, and Romans learned to concentrate fermentation alcohol by distillation so it could be utilized for industrial as well as human consumption. Early industrial uses were for perfumes, cosmetics and medicines. By the mid-seventeenth century distilleries appeared in Europe with improved technology, and ethanol became an important chemical raw material, especially during the Industrial Revolution (1,2,3).

Until about forty years ago industrial ethanol in the United States was fermented predominantly from cane molasses from Cuba, but subsequently from three grains: corn, wheat, and sorghum. Fermentation ethanol was then displaced by chemically synthesized ethanol from petroleum derived ethylene. In 1976 over 98% of the industrial ethanol in the U.S. was produced from ethylene. However, fermentation ethanol is now once again the dominant source of ethanol in the U.S. In 1981 in the U.S., 185 million gallons of synthetic ethanol and 230 million gallons of fermentation ethanol were produced (5). Fermentation ethanol capacity in the U.S. is expected to increase by 200 million gallons per year by the beginning of 1983 from a fermentation ethanol capacity of

about 425 million gallons per year and a synthetic ethanol capacity of 271 million gallons per year in mid 1982 (5).

The present major uses of ethanol industrially are in acrylate polymers production and in fragrance solvents. Fermentation ethanol has also recently received interest as a liquid fuel since gasoline availability has fluctuated. As ten percent of an ethanol-gasoline mixture called gasohol, ethanol may serve to extend the gasoline supply and increase its octane (4).

Despite the long history of alcoholic beverage production, its production technology is not directly applicable to industrial alcohol fermentation. High capacity and low cost ethanol production require low cost raw materials, high ethanol yields, high ethanol concentrations, and high production rates, which are not important factors in beverage making. The present industrial alcohol fermentation technology predominantly in practice is that developed before the 1940's (6). However, higher productivity continuous processes and lower energy recovery processes are currently being developed to reduce capital and operating costs (6,7,12,18,19,29). Simultaneously there has been basic and applied research on the biological properties of yeast to understand and remove the biological limitations on ethanol productivity (18,20,21,22).

1.2 Research Objectives and Rationale

Economic evaluations of both traditional and current processes show that ethanol production costs are dominated by the raw materials costs, which are primarily associated with the sugar cost (6,7,9,10,12). Complete and rapid utilization of the sugar with a maximum yield of ethanol

is imperative to low ethanol costs. This utilization is dependent on the properties of the fermenting organism, the feed medium, and the fermentation environment. However, feed medium optimization in general has been qualitative in nature with arbitrary additions of various substrates (8). Important nutrient effects have been masked by use of complex (chemically undefined) media (8). The effects of limiting concentrations of most substrates and, especially growth factors and trace elements, have not been systematically studied and well understood with respect to ethanol production. Furthermore, inhibitory effects from high concentrations of certain feed components and by-products are important aspects of the environment which have not been well studied.

The general objectives of this research are to select an ethanol producing organism as a model system, optimize the feed medium, and optimize the fermentation environment. In addition to providing practical bases for formulating media and conditions, an attempt will be made to provide a conceptual understanding of the effects of the medium components and environment on cell growth and ethanol production.

More specifically, the organism selected ideally should be thermophilic, ethanol tolerant, easily separated, non-pathogenic, high ethanol yielding, rapid ethanol producing, non fastidious in nutritional requirements, stable with respect to mutation, suitable for industrial fermentation, and similar in metabolism to other likely ethanol producing candidates, for which it can serve as a model. The environmental factors in the fermentor to be studied are temperature, dissolved oxygen, carbon dioxide, ethanol, and inhibitory concentrations of glucose and other feed components. The emphasis of this work, however, is on

media optimization. Feed media should be optimized for varied sugar sources, minimal costs, minimal inhibition, and various process conditions; the rationale for these conditions will be discussed in the following subsections.

1.2.1 Media Suitable for Varied Sugar Sources

Ethanol can be fermented from diverse feedstocks. The most common sugar substrates are sugar cane juice and molasses. Starches from grains (corn, rice, wheat, etc.) and from root plants (cassava, sweet potatos, etc.) can also be fermented after enzymatic hydrolysis to simple sugars. In addition, considerable research and development are presently directed toward production of fermentable sugars economically from renewable and waste cellulosic sources, such as trees, agricultural residues and municipal wastes. The raw material prices, ethanol conversion yields and contributions to ethanol production costs for various feedstocks are given by Maiorella et al. (6).

Because of the diversity of complex feedstocks, it is impractical to optimize each case empirically. Rather, a general approach will be taken by using synthetic (chemically defined) and semi-synthetic (partially undefined) media to determine the optimum concentrations of all major active components of the medium. Given these individual component requirements and the feedstock composition of these components, the feedstock can in principle be supplemented or depleted to approximate the chemically defined optimum medium. Thus, optimum media can be formulated for a wide variety of sugar sources from a limited optimization study using defined medium components. Glucose, the major sugar in the most widely used feedstocks, is the only sugar considered in these

studies.

1.2.2 Minimal Cost Media

During the initial stages of fermentation process development, nutrients rich complex media are normally used to determine the maximum productivity of the process without media limitations. Cysewski (9) used the high yeast extract medium given in Table 1.1 to obtain maximum cell and ethanol productivity with the same yeast used in this study. Based on the costs given in Table 1.2, the 8.5 g/L yeast extract in this 100 g/L glucose medium would contribute 8.5 x $2.98/gal = $25.33/gal to the ethanol production cost. Even the least expensive form of yeast, Amber BYF 300 autolysate, in the ratio of 8.5 g/L to 100 g/L glucose would cost $0.96/gal ethanol. For reference, the current ethanol selling prices are $1.70/gal, 190 proof, and $1.82/gal, 200 proof tax free (5). The other components in the medium used by Cysewski (9) contribute only a few cents per gallon ethanol produced.

The cost of Cysewski's medium with glucose as the sugar source and yeast extract as a complex supplement represents the worst case, for a feedstock containing no nutrients other than sugar. Cellulose hydrolysates are normally low in nutrients content. Table 1.3 shows Wilke et al.'s (10) projection of the medium chemicals needed to supplement glucose from corn stover hydrolysate and their updated costs. Even with only 2.86 g/L Amber BYF-300 yeast autolysate per 100 g/L glucose, this autolysate adds $0.323/gal to the ethanol cost. With more commonly used starch and sugar feedstocks, such as corn and molasses, the medium requirements should be much lower.

Table 1.1

High Yeast Extract Concentration Medium used by Cysewski (9)

Component	Concentration (g/L)
Glucose (J.T. Baker reagent)	100.0
Yeast Extract (Difco)	8.5
NH_4Cl	1.32
$MgSO_4 \cdot 7H_2O$	0.11
$CaCl_2$	0.06
Antifoam (General Electric AF 60)	0.2 ml
Tap Water	Make up to 1 liter

Table 1.2

Forms and Costs of Yeast Extract and Yeast Autolysates

Yeast Form[1]	Supplier	Unit Cost[2] $/lb	Cost of Ethanol[3] $/gal/g/ 100 g-glucose
Yeast Extract "Difco Certified"	Difco Lab. Detroit, MI.	21.38	2.98
Yeast Extract Amber prymex 215, debittered	Amber Lab. Milwaukee, WI.	1.90	0.26
Yeast Extract Amber BYF 1003, slightly bitter	Amber Lab. Milwaukee, WI.	1.74	0.24
Yeast Autolysate Amber BYF 100, centifuged	Amber Lab. Milwaukee, WI.	1.10	0.15
Yeast Autolysate Amber BYF 300, unseparated	Amber Lab. Milwaukee, WI.	0.81	0.11

[1]All forms of yeast are spray dried.

[2]Costs are from Spring, 1982.

[3]Cost of ethanol per gallon is per gram of yeast extract or autolysate per 100 grams of glucose feed with an ethanol yield of 0.47 g-ethanol/g-glucose consumed.

Table 1.3

Medium Chemicals for Ethanol Fermentation of Corn Stover Hydrolysate (10)

Component[1]	Unit Cost[2] $/ton	Conc. (g/L)	Cost of Ethanol ($/gal)
Glucose from corn stover		100.	
$(NH_4)_2SO_4$	65.	1.86	0.0084
$MgSO_4$	304.	0.08	0.00169
$CaCl_2$	154.	0.08	0.00086
Protein Nutrient[3]	1620.	2.86	0.323

[1] Trace elements are assumed supplied by process water.

[2] Unit costs are updated to March, 1982.

[3] Amber BYF-300 from Amber Laboratories, Milwaukee, Wisconsin.

To decrease ethanol production costs, the two most important costs to reduce are for sugar and for yeast extract or autolysate. Reduction of sugar costs may come from improved cellulose conversion processes (11,12,13,14) but is beyond the scope of this study. The yeast extract or autolysate costs can be reduced by determining their active components and substituting less expensive sources or reducing the requirements for these components. For example, potassium, phosphorous, vitamins, and various trace elements are not explicitly present, but are in the form of yeast extract or autolysate in the media of Tables 1.1 and 1.3. Many of these nutrients could be less expensively supplied as bulk or pure chemicals. For required, but unidentifiable components of complex factors, less expensive substitutes, such as corn steep liquor, should be investigated. The corn steep liquor cost is $0.055/lb, F.O.B. Argo, Il. in 90,000 lbs. tank car quantities (15).

Determining the requirements for balanced media will reduce excess chemicals cost. The media requirements for growth factors and unidentified complex components may also be reduced by long term continuous culture adaptation to minimal media deficient in these factors. After the medium has been defined in terms of the concentrations of the active nutrient components, linear programming can be used to optimally blend the available raw materials of known composition to minimize raw materials costs within nutrient requirement constraints.

1.2.3 Minimal Inhibition Media

Continuous ethanol fermentation processes in which ethanol is simultaneously and selectively removed are being developed to reduce ethanol inhibition of cell and ethanol productivities. Selective

ethanol removal processes include vacuum stripping, extraction and membrane separation of the fermentation broth. In such systems secondary by-products and unutilized feed components can build up to inhibitory concentrations (16,17). Toxic build up of the same components can also occur with slopping back, i.e., recycle of stillage from the distillation bottoms back to the fermentor.

To reduce these inhibitory effects in fermentations coupled with ethanol removal, a bleed of the fermentor broth must be maintained. For the relative feed component concentrations in Table 1.1 with 334 g/L glucose in a vacuum fermentation without recycle, Cysewski's (9) feed to bleed ratio was 3.1 at 80% inhibition of cell productivity. This large bleed stream reduces cell and ethanol productivity and increases waste treatment costs. In slopping back, stillage as a per cent of the fermentor broth is limited to 10 - 20% for blackstrap molasses (24,27), 20 - 25% for corn (28), and up to 50% for high test molasses (24). Alfa-Laval (25,26) found toxicity problems from concentration of feed components in their Biostil process.

Maiorella et al. (17) showed that all the major salts in the feed to yeast become increasingly toxic at high concentrations. Excess feed salts must be minimized to reduce these forms of substrate inhibition. Therefore, determining a balanced medium is an important objective of this study for both process and economic reasons. For process purposes, a feed medium can be considered balanced if its components are not the limiting inhibitory factors to higher feed to bleed ratios or to more stillage recycle. Formulation of balanced media will be facilitated by using synthetic and semi-synthetic media to determine the requirement

for each active component.

The residual sugar concentration in continuous stirred tank fermentors is controlled at negligibly low levels by the dilution rate. However, the inlet concentration for plug flow and the initial concentration for batch fermentors are the feed concentrations. High sugar feed concentrations are necessary for high ethanol concentrations, but can also cause inhibition. Glucose inhibition of growth and ethanol production is significant at 100 - 150 g/L and total inhibition is attained at 350 - 500 g/L (23). This glucose inhibition effect will be studied in batch fermentations with different initial glucose concentrations. The initial rates of cell growth and ethanol production show the glucose inhibition effect without significant ethanol inhibition.

1.2.4 Media for Various Process Conditions

The fermentor process determines the fermentation parameters to be optimized by the media and environmental conditions.

For single stage continuous stirred tank processes, the important parameters to be maximized are ethanol yield (g ethanol/ g glucose consumed) and ethanol productivity (g ethanol/ L-hr). High ethanol productivity in this case requires high cell mass productivity. The dilution rate (reciprocal of fermentor residence time) is adjusted to fully utilize the sugar.

For high cell density fermentors, such as cell recycle and immobilized cell systems, ethanol yield and specific ethanol productivity (g ethanol/ g cell-hr) are the parameters to be maximized. Total glucose consumption is assured by high cell densities, achieved through cell

recycle or immobilization. Total ethanol productivity is the product of the controlled cell mass concentration and the specific ethanol productivity.

The effect of feed concentration of each major component in the medium will be studied in continuous culture with respect to the following parameters: ethanol and cell mass concentrations, ethanol and cell mass yields, specific ethanol productivity, and fraction glucose utilization. These individual nutrient effects will provide the bases for formulation of media optimized for the desired parameters of a given process.

In fermentation systems without simultaneous ethanol removal, the feed sugar concentration is also limited by ethanol inhibition. To determine the effect of ethanol inhibition on media requirements, they will be determined for both low (10 g/L) and high (100 g/L) glucose feed concentrations. The low glucose studies will be carried out with and without ethanol added to the feed. These studies should also separate the effects of nutritional limitations from ethanol inhibition limitations on ethanol productivity.

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Chapter 2

2. Background and Previous Work

2.1 Media Requirements

Saccharomyces cerevisiae is a facultative anaerobe, which requires an organic substrate, usually a sugar, for both carbon and energy sources under fermentative and oxidative conditions. The organic substrate is also the main source of oxygen and hydrogen to the cell. In addition, nitrogen, potassium, phosphorous, sulfur, magnesium and calcium are required in major quantities. These elements are important structurally as parts of cell components and catalytically as cofactors. Minerals required in much lower concentrations are designated trace elements and include boron, cobalt, copper, zinc, manganese, iodine, iron and aluminum. Their low concentrations indicate trace elements are required in catalytic roles, but many of their exact functions are not understood. Growth factors, consisting of vitamins and organic precursors, are also required in very low concentrations. Vitamins serve mainly as coenzymes; their functions are described in Chapter 7. In this chapter the forms and concentrations of these nutrient requirements used in industrial ethanol production and previous research studies are discussed.

2.1.1 Carbon Source

Yeasts are able to ferment only the D isomer forms of sugars (1, 2). The hexoses, D-glucose, D-fructose and D-mannose, are usually the only fermentable forms of monosaccharides (1, 2). Pentoses are not

usually fermented (1, 3); moreover, pentoses, especially xylose, inhibit brewer's but not baker's yeast fermentation of hexoses (1, 4). Exceptions are Pachysolen tannophilus, which can ferment D-xylose to ethanol (84), and strains of Schizosaccharomyces pombe, Kluyveromyces lactis and Saccharomyces cerevisiae, which can ferment D-xylulose to ethanol (85). Galactose can be fermented by brewer's and baker's yeast after adaptation (1, 5).

Among the disaccharides, sucrose is hydrolyzed to glucose and fructose by invertase enzyme and then fermented by most yeasts. Maltose fermentation requires adaptation (1, 6, 7) but may be faster than glucose fermentation by baker's and brewer's yeast (1, 8, 9). Cellobiose, a major by-product of cellulose hydrolysis, is not fermented by brewer's or baker's yeast (10, 11). Candida blankii, Candida inositophila, Candida lusitaniae and Pichia wickerhamii, however, can ferment D-cellobiose and D-xylose to ethanol (86). Barnett (12) reports only 3 out of 439 species of yeast tested were able to ferment lactose. Izaguirre and Castillo (13) found seven strains of Candida pseudotropicalis and two strains of Kluyveromyces fragilis capable of significant ethanol production from 7 % (w/v) whey. Trehalose is fermented, but at low rates by baker's and brewer's yeast (14, 15, 16).

Among the oligosaccharides, raffinose was found fermentable by 47 out of 439 yeast species by Barnett (12). None of these species were able to utilize inulin or starch (12).

Under oxidative conditions, yeasts can utilize the same sugars for respiration as for fermentation under anaerobic conditions. Furthermore, they can metabolize for growth non sugar substrates, such as,

ethanol, acetic acid, dihydroxyacetone, α-ketoglutaric acid and oxaloacetic acid (17, 18, 19).

2.1.2 Nitrogen Source

Approximately 10 % of the yeast dry weight is composed of nitrogen. Its concentration requirement as a nutrient in media is second only to the carbon source. The nitrogen source can either be inorganic, as ammonium salts, or organic, as amino acids, peptides, urea, purine and pyrimidine bases.

The ammonium salts can be utilized in the form of sulfate, phosphate, bicarbonate, carbonate, acetate, lactate, tartrate and chloride (1). Pirschle (20) found diammonium phosphate best for growth; the chloride form was considered worst among the above ammonium salts (1). However, the equilibrium concentration of ammonium ions for all forms should be approximately the same at a fixed pH. Nitrates can be assimilated by Candida utilis but not by brewer's or baker's yeast (1).

Thorne (21) ranked the nutritional values of amino acids as sole nitrogen sources for yeast growth without aeration as shown in Table 2.1. The relatively higher values for asparagine, aspartic acid and glutamic acid with respect to ammonium phosphate indicate these amino acids are functionally more than simply nitrogen sources, and possibly, are direct precursors to protein according to Thorne (22). Suomalainen and Oura (1) claim that an ammonium salt is a better nitrogen source than any amino acid alone, but in mixtures of amino acids and ammonia nitrogen, the uptake rate of amino nitrogen is much faster (1, 23). Jones et al (24) show that amino acids are not directly assimilated, but

Table 2.1

Amino Acids as Sole Nitrogen Source for Yeast Growth

Amino Acid	Relative Nutritional Value (from Thorne (21))
Mixed amino acids in grain wort	150 - 200
Asparagine	142
Aspartic acid	128
Glutamic acid	104
Ammonium phosphate	100
Arginine	87
Leucine	87
Valine	78
Proline	69
Alanine	68
iso-leucine	65
Methionine	64
Phenylalanine	64
Serine	59
Tyrosine	58
Tryptophane	49
Hydroxyproline	42
Histidine	31
Cystine	22
Glycine	15
Lysine	8

first go through reactions, such as transaminations. Peptides vary in degree of assimilation by yeast. Amino acids and peptides also have chelating properties, which can complex or precipitate inhibitory medium components, such as heavy metals (23, 25).

Among the other nitrogen sources, urea with adequate biotin in the medium is equivalent to ammonium sulfate in promoting growth (26, 27). Utilization of purines and pyrimidines for growth by Saccharomyces species varies widely (1).

2.1.3 Major Minerals

Potassium, magnesium, phosphorous and sulfur are minerals essential to growth and required in major quantities. Calcium is required in lower concentration and appears to be a stimulant to growth (33). Calcium storage in the cells may, however, be masking its requirement as an essential element (32). Also, magnesium can substitute in large part for calcium (34). The catalytic and structural roles of the major minerals are described in Chapter 7.

The salt form of potassium, magnesium and calcium has not been shown to be important, but is commonly a chloride or sulfate. Phosphorous is required as a phosphate; Rothstein (28) reports that yeasts absorb the monovalent anion $H_2PO_4^-$, but not the bivalent anion HPO_4^{-2}. More $H_2PO_4^-$ can be absorbed as a potassium than as a sodium salt (28).

Uptake of potassium, magnesium and phosphate are interdependent. Rothstein (28) noted that cells rich in potassium have a much higher uptake of phosphate than cells poor in potassium because of the need to maintain an acid-base balance in the cell. The magnesium uptake rate

depends on the phosphate uptake because the activity of the magnesium carrier is induced only with transport of external phosphate through the cell membrane, suggesting phosphate is required for part of the Mg carrier system (28).

Sulfur can be utilized in inorganic form as sulfate or in fewer cases as sulfite or thiosulphate (29, 30, 31). Usable organic forms of sulfur in general order of preference are methionine, glutathione and cysteine (23, 29, 32).

2.1.4 Trace Elements

Concentrations of trace elements are much lower and requirements are less well known than those for the major minerals. In many cases the requirements can not be demonstrated because of their presence as contaminants of other media components (25). Knowledge of the functions of the trace elements in the cell should help determine their requirement as essential or stimulatory factors. These functions are expected to be mainly catalytic, as cofactors for enzymes. Many of the trace elements which are required for growth at low concentrations can be inhibitory at high concentrations.

In a survey of the trace metal composition of five baker's yeast and five brewer's yeast, Eddy (35) found the following elements (with the number of strains found in enclosed by parenthesis): aluminum (5), barium (5), boron (1), chromium (1), cobalt (5), copper (5), iron (5), lead (7), manganese (9), molybdenum (3), nickel (3), tin (4), vanadium (3), zinc (6). Presence of a trace element in a cell does not necessarily show requirement for it. Trace elements with reported positive

effects on growth or fermentation activity are given in Table 2.2.

2.1.5 Growth Factors

Growth factors consist mainly of vitamins, which are cofactors for enzymatic reactions, and organic precursors, which promote cell growth. As with the trace elements, requirements of growth factors vary widely among yeast. Therefore, the requirements for individual strains must be determined experimentally. Even so, the growth factor requirements may change as the yeast adapts to its medium (40).

As a starting point, the following growth factors in the analyses of 10 yeasts (2 brewer's, 2 baker's, 2 Candida utilis, 2 Kluyveromyces fragilis, 2 Candida lipolytica), as reported by Reed and Peppler (41), can be considered: biotin, pantothenate, thiamine, pyridoxine, inositol, riboflavin, nicotinic acid, folic acid, p-aminobenzoic acid, and choline. D-biotin is the single most commonly required growth factor (41, 42, 43); its absence also appears to have the greatest effect on cell growth (23, 41, 42). Alternate forms of D-biotin which are as effective are D-biotin methyl ester, biocytin, biotin D-sulphoxide, and D-desthiobiotin (1). Suomalainen and Keranen (44) reported that oleic and palmitoleic acids with aspartic acid could substitute for biotin in baker's yeast under aerobic conditions. (The D-isomer of biotin is assumed if not specified).

Inositol, myo-inositol and meso-inositol are different names for the same optically inactive stero-isomer. The D- and L- forms are inactive as growth factors (45). Inositol appears to function as both a cofactor and as a precursor for lipid structures. Hence, its

Table 2.2

Trace Elements with Known Functions and Ionic Forms

Ion	Function	References
Fe^{2+}, Fe^{3+}	Essential for catalase and cytochrome activity; inactivates yeast proteinases; stimulates fermentation and growth	32
Zn^{2+}	Essential for glycolysis and selected vitamin synthesis; part of metallo-enzymes, such as cysteine desulphydrase	23,58
Cu^{2+}	Affects redox potential; increases activity in cytochromes and in citric acid cycle	36, 37, 38
B^{2+}	Stimulates growth at low concentration	23
Mn^{2+}	Involved in activation of arginase, regulation of Zn^{2+} effects; stimulates protein utilization	32, 23
Co^{2+}	Can enhance activity of aldolase, phosphoglucomutase and peptidase (other ions can substitute for Co^{2+}); stimulates protein utilization	32, 58
I, I^-	Stimulates growth	39
Cl^-	Appears essential to growth; counter ion to movement of some positive ions	32, 23

concentration requirement is much greater than that for most vitamins.

Thiamine and pyridoxine are commonly required growth factors, but for some yeasts, particularly brewer's top yeast, either one is sufficient (1, 46, 47). In other yeasts, thiamine inhibited cell growth, but addition of pyridoxine could alleviate the inhibition (84). Thiamine in the cell is usually in the form of diphosphothiamine or cocarboxylase (47). Rahn (48) reported that thiamine imparted ethanol tolerance to yeast. Thiamine stimulated the fermentation rate of baker's yeast according to Schultz et al (49). Pyridoxine is one of three naturally occurring forms of vitamin B_6, the others being pyridoxol and pyridoxamine. These other two forms can only partly replace the pyridoxine requirement in some strains of Saccharomyces cerevisiae (50).

Pantothenic acid is important as a component of coenzyme A, but yeasts requiring pantothenic acid do not respond as well to coenzyme A and other conjugates, perhaps, because of cell wall permeability problems (51). β-alanine, which is a component of pantothenic acid, is an effective substitute for many but not all yeasts (43, 51).

According to Suomalainen et al (52), nicotinic acid (commonly known as niacin) is required as a growth factor to supplement its limited production by baker's yeast under anaerobic conditions. Nicotinic acid and nicotinamide appear equally effective for yeast (53, 54); either can become constituents of the coenzyme, nicotinamide adenine dinucleotide, which can also satisfy the growth factor requirement (54).

Since riboflavin and folic acid can usually be synthesized by all yeasts (1), they are seldom required as growth factors. Para-

aminobenzoic acid is required by only a few strains of yeast, including some strains of brewer's yeast (1, 43). Part of the function of p-aminobenzoic acid is in biosynthesis of folic acid (55), but its growth factor requirement is probably also for less certain functions in the biosynthesis of vitamin B_{12} (55) and pantothenic acid (51).

The choline content is of the same order as the inositol content of baker's and brewer's yeast, i.e., 2 - 5 mg/g (35). Choline also is incorporated into lipid as phosphatidylcholine, but relatively few yeasts are auxotrophic for choline. Kortsee (56) showed that none of 64 yeast strains, including 6 Saccharomyces strains, could utilize choline in the medium. These yeasts may be not be able to degrade or transport choline into the cell (57).

2.2 Industrial Ethanol Fermentation Media

2.2.1 Corn (59, 60)

Corn is presently the major starch based feedstock used for fermentative ethanol production in the United States. After cooking the corn to break the cell walls and to gelatinize the starch, the starch can be hydrolyzed to fermentable sugars by barley malt or by fungus produced enzyme. As predominantly used in the United States, barley malt constitutes 8 to 10 % of the corn mash. The barley malt also provides most of the nutrients in the corn mash; nevertheless, this mash is marginal in nutrients content. Therefore, stillage from the distillation bottoms product is added to make up 20 to 25 % of the mash to be fermented. The stillage provides nutrients, reduces the pH to the optimum initial level (from 4.8 to 5.0), and provides pH buffering between 3.5 and 4.4. After

adjustment with stillage and/or water, the total sugar concentration of the mash is about 130 g/L as glucose. The inoculum is 2 % of the final fermentative volume and is grown semi-aerobically in a 70 % corn and 30 % barley malt mash, to which 20 % stillage by volume and 0.0053 lb urea/gal mash are added.

An approximate analysis of the corn mash medium can be obtained from the compositions of corn stillage, corn meal, barley, and dried malt extract in Table 2.3. The fermentation mash can be assumed to consist of 1) 20 % stillage, which is 7 % total solids with the dry composition given by "dark grains," 2) 68 - 72 % corn, and 3) 7.5 % - 8 % barley malt. The "dark grains" are produced by combining the dried "light grains," which come from simple screening of stillage, and "distillers' solubles," which is the evaporated and dried thin stillage with soluble components.

2.2.2 Molasses (63, 64)

Molasses is one of the most important sugar containing feedstock in fermentative ethanol production. Common types of molasses for fermentation are 1) blackstrap derived from the mother liquor of cane juice from which sucrose is obtained by three evaporation and crystallization steps and 2) high-test derived from evaporation of the cane juice with partial hydrolysis of, but not crystallization of sucrose. Blackstrap molasses contain more nutrients, but also more unfermentable reducing substances from decomposition of sugars than high-test molasses. The compositions of these molasses are compared in Table 2.4.

The main fermentation mash or medium is prepared by diluting the

Table 2.3

Composition of Corn Mash Components

	Corn Stillage "Dark Grains"	Corn Meal (Maize)	Barley	Dried Barley Malt Extract
References	(59, 61)	(62)	(62)	(62)
Moisture (%)	5	10.8	15	2.0
Protein (%)	27	10.0	10	4.6
Fat (%)	7	4.3	1.5	
Fiber (%)	2	1.7	4.5	
Ash (%)	8	1.5	2.6	1.5
Carbohydrate (%)	51	71.7	66.4	91.9
Maltose				52.2
Glucose				19.1
Sucrose				1.8
Dextrin				15.0
Other carbohydrates				3.8
Growth Factors (p.p.m.)				
Thiamine	8	4.5	6.5	
Riboflavin	22	0.9	1.2	
Pantothenic acid	29	4.6	4.4	
Nicotinic acid	125	23	115	
Pyridoxine	9	6.9	11.5	
Biotin	0.5	0.1		
Choline	6500		1100	
Carotene	0.8			
p-Aminobenzoic acid	10			
Folic acid	4			
Zeaxanthin	8			
Cryptoxanthin	4			

Table 2.4

Composition of Blackstrap Molasses, High-test Molasses and Molasses Stillage

	Blackstrap Molasses	High-test Molasses	Molasses Stillage
References	(62,63,67,68)	(62,63,68)	(63)
Solids (%)	83-85	80-85	8.5
Sucrose (%)	30-40	15-35	
Invert sugar (%)	12-18	40-60	
Ash (%)	7-10	2-4	2.0
Organic nonsugars (%)	20-25	4-8	
Fermentable sugars as % of total sugars	90	95	
Sugars (copper-reducing substances) (%)			1.0
Proteins (%)			1.3
Gums (%)			2.0
Glycerol (%)			0.49
Lactic acid (%)			0.51
Fiber (%)			0.056
Wax, lignin, glucosides, phenolic bodies, organic acids, etc. (%)			1.2
N (%)	0.7	0.05-0.25	
P (%)	0.9	0.03-0.3	0.06
Ca (%)	0.5	0.10-0.25	0.26
Mg (%)	0.07	0.02-0.15	0.1
K (%)	3.6	0.2-0.9	0.64
Si (%)		0.03-0.11	0.04
Al (%)		0.001-0.005	0.02
Fe (%)		0.0007-0.003	0.02
Cu (%)			0.003
C (%)		28-36	
S (%)			0.26
Cl (%)			0.24
Mn (%)			0.0004
I (%)			0.00026
Thiamine (ppm)	8.3		
Riboflavin (ppm)	2.5		2
Pantothenic acid (ppm)	21-40		7.3
Nicotinic acid (ppm)	21-30		3.9
Pyridoxine (ppm)	6.5		5.6
Biotin (ppm)	1.2-3.2		0.28
Inositol (ppm)	4000		
Folic acid (ppm)	0.038		0.06

molasses to a sugar concentration of 14 to 18 % with water. Blackstrap molasses normally does not require additional new nutrients, but in some cases 0.5 to 3 pounds of ammonium sulfate per 1000 gallons of mash are added to increase the ethanol production rate and ethanol yield from sugar fermented. Additional nutrients are provided with "slopping back" of stillage from a previous fermentation up to 10 to 20 % of the mash volume for blackstrap molasses. High-test molasses is supplemented by 3 to 6 lbs ammonium sulfate per 1000 gal mash, about 3 lbs phosphoric acid per 1000 gal mash, and up to 50 % of the mash volume by stillage. An analysis of molasses stillage is given in Table 2.4. An inoculum grown on 8 to 12 % sugar provides 2 to 4 % of the main fermentation mash by volume. Sulfuric acid (1 - 2 gal/1000 gal mash) is used to adjust the pH; an initial pH between 4.8 and 5.0 is considered optimum. Aqua ammonia is used during fermentation of high-test molasses to control pH.

2.2.3 Sulfite Waste Liquor (66)

Sulfite waste liquor is the waste product from the sulfite pulping of wood to pulp for paper making. The liquor is an aqueous solution of sulfurous acid, cations (calcium, magnesium, sodium, or ammonium), lignin, hemicellulose, organic acids, sugar decomposition products, and fermentable sugars. Lignin sulfonates constitute about 65 % of the dissolved solids. An approximate partial composition of sulfite waste liquor is given in Table 2.5.

The sulfite waste liquor is prepared for fermentation by simultaneous neutralization and removal of fermentation inhibiting sulfur dioxide derivatives and bisulfite addition compounds, using a combination of aeration, steam stripping, and lime addition. Using about 3 lbs lime per

Table 2.5

Composition of Sulfite Waste Liquor (62, 66)

Total solids	10 - 12 % w/v
Solids in suspension	5 - 15 % of total solids
Ash	1.0 - 2.1 % w/v
Calcium oxide	0.62 - 0.96 % w/v
Free sulfur dioxide	0.06 - 0.80 % w/v
Loosely combined sulfur dioxide	0.37 - 0.62 % w/v
Sulfate, as sulfur trioxide	0.11 % w/v
Lignin sulphuric acid	7.8 % w/v
Total sulfur	0.78 - 1.58 % w/v
Methoxyl	0.78 - 0.81 % w/v
Fermentable sugars, as glucose	1.25 - 1.91 % w/v
Total reducing substances, as glucose	2.05 - 2.7 % w/v
Mannose	0.8 % w/v
Xylose	0.4 % w/v
Galactose	0.1 % w/v
Arabinose	0.7 % w/v
Glucose	0.1 % w/v
pH	3.8 - 4.2

1000 gal sulfite waste liquor, pH is adjusted to 4.5 for fermentation. The only supplementary nutrients required are usually ammonia, ammonium hydroxide or urea. Yeast is recovered for subsequent fermentation by centrifuging part of the fermentor slurry to a yeast concentration of about 15 % by volume. Joined by the clarified stream of about 0.02 % yeast from the centrifuge, another part of the fermented liquor goes directly to distillation. This bypass prevents toxic accumulations in the fermentor. McCarthy (66) and Aries (59) do not report use of stillage recycle for sulfite waste liquor.

2.2.4 Limitations of Industrial Media

Although most major feedstocks are rich in many nutrients, not all components are utilizable. White (65) notes that yeast can only assimilate about half of the phosphates in molasses. Certain forms of inorganic and organic nitrogen, such as betaine, also can not be assimilated by yeast (62).

The composition and quality of complex raw materials vary with the producer and with time for the same producer (62). Despite pasteurization, barley malt is the main source of bacterial contamination of grain mashes (60). Alcohol yields are affected by the number and type of bacteria in the malt.

The mash may require extensive pretreatment for pH adjustment and/or inhibitors removal. Grain and molasses mashes require pH adjustment, using sulfuric acid and stillage. Waste sulfite liquor requires aeration, steam stripping, and lime for reducing concentrations of sulfur dioxide and other toxic substances and for increasing the pH.

Inhibitory products and loss of substrates may result from heating complex mixtures of reducing sugars, amino acids and phosphates (69, 70). This heating occurs during production of molasses, resulting in non fermentable caramels, melanoidins, hydroxymethylfurfural, acetoin, formic acid, levulinic acid, and other decomposition products (63). In fermentation of corn, the malt slurry is prepared at 145 ^{o}F and the stillage is heated to 220 - 225 ^{o}F (60). At these temperatures growth factors may also be degraded.

The major problem with unbalanced complex media occurs with toxic buildup of unutilized feed components (71) and by-products (72). This accumulation takes place in fermentors with selective ethanol removal and in fermentors with stillage recycle. The results are greater bleeds and less recycle, causing lower ethanol productivity and higher waste production.

Concentration of certain minerals in the stillage reduces its utility and value. Molasses stillage, which is especially high in potassium, can only be used for up to 10 % of ruminant diets and up to 2 % of pig diets (61) because of its laxative effects. Long term application of stillage as a fertilizer results in accumulation of sulfates, which are reduced to hydrogen sulfide and then reoxidized to sulfuric acid in the soil.

The complex nature of industrial media does not allow well controlled studies of medium component effects. The relative productivities and yields of ethanol and cell mass depend on the ratios of the feed components, which are not easily varied in complex media. Important nutritional effects can also be masked by complex media (25).

2.3 Research Studies of Media Requirements

2.3.1 Media and Limitations

Oura (73) summarizes ten of the most well known media for yeast growth on a common basis of 200 grams glucose and all synthetic components, assuming given compositions for complex components. These media were formulated for aerobic growth of 100 grams of yeast. The non-glucose media requirements for fermentative growth can be approximated as 10 to 20 % of the aerobic requirements and then scaled to the desired glucose concentration for fermentation. This fermentation medium may support the desired growth level, but may not be optimal for high ethanol production rate and yield. Furthermore, concentration requirements of some components in Oura's (73) set of media vary more than an order of magnitude, reflecting in part the dependence of certain requirements on specific strain.

Nagamune et al (74) carried out the most systematic study that could be found of nutrient effects on ethanol production by yeast with synthetic media. They studied the effects of five groups of nutrients in shake flasks with 5.0 g/L initial glucose concentration:

(1) Nitrogen sources - asparagine and $(NH_4)_2SO_4$

(2) K^+ and P source - KH_2PO_4

(3) Mg^{2+} and Ca^{2+} sources - $MgSO_4$ and $CaCl_2$

(4) Zn^{2+}, Fe^{2+} and Cu^{2+} sources - $ZnSO_4$, $FeSO_4$ and $CuSO_4$

(5) Vitamin sources - inositol, thiamine HCl, pyridoxine HCl, biotin, Ca-pantothenate, nicotinic acid and p-aminobenzoic acid

Each group was studied at three concentrations for a total of $3^5 = 243$ combinations, which include all main and interaction effects. Maximum specific rates of glucose consumption, ethanol production and cell growth were measured. However, only relative specific rates (normalized by the rates for the basal medium) are reported as functions of relative nutrient concentrations. The minimum concentration ratios of nutrients to glucose, as functions of glucose concentration, to maintain the maximum specific rates are also presented.

The utility of this study is limited by the absence of absolute rates, the lack of data for many effects reportedly studied, and the failure to separate individual effects within many nutrient groups - especially the vitamin group. Furthermore, nutrient studies in batch culture are extremely dependent on the inocula culture media and conditions, which are not specified. Accurate specific ethanol production and glucose consumption rates are also normally difficult to obtain in batch cultures.

Haukeli and Lie (75) compared batch growth curves for anaerobic fermentations carried out with synthetic and semi-synthetic media of vitamins, amino acids, vitamin free casamino acids, yeast extract, ergosterol and trace oxygen. Increased growth rate of Saccharomyces carlsbergensis was observed with successive addition of each of the following factors: biotin, pyridoxine, thiamine, the set of folic acid, Ca-pantothenate, inositol, nicotinic acid, p-aminobenzoic acid and riboflavin. Ergosterol increased the growth rate and final cell concentration for anaerobically grown inoculum; yeast extract further increased growth rate and final cell concentration relative to the set

of nine vitamins given above. Casamino acids (0.1 %) added to a complete vitamin medium increased the final cell concentration of Saccharomyces cerevisiae close to that for 0.3 % yeast extract medium. Ethanol production and glucose consumption data are not presented.

Leonian and Lilly (41), Schultz and Atkin (76), and Wiles (77) surveyed the growth factor requirements of 31 Saccharomyces cerevisiae strains as summarized in Table 2.6. Atkin et al. (42) determined the growth factor requirements of 58 brewer's yeast - 53 lager or bottom yeasts and 5 ale or top yeasts - as given in Table 2.7.

Among semi-synthetic media with relatively low concentrations of complex factors, Wada et al.'s (78, 79) medium was able to support long term continuous ethanol production with high ethanol yield and productivity in an immobilized yeast column. This medium consisted of 10 or 25 % glucose, 0.15 % yeast extract, 0.25 % NH_4Cl, 0.55 % K_2HPO_4, 0.025 % $MgSO_4 \cdot 7H_2O$, 0.1 % NaCl, 0.001 % $CaCl_2$ and 0.3 % citric acid (pH 5.0).

Saccharomyces cerevisiae OUT 7013 could ferment this medium with 10 % glucose to 50 g/L ethanol at a residence time of 1 hr for more than 3 months. With the same medium, Saccharomyces cerevisiae IFO 2363 could ferment 25 % glucose (stepped up in 5 % increments from 10 % every 6 days) to a final steady state ethanol concentration of 114 g/L at a residence time of 2.6 hr for over 2 months.

The high ethanol yield and productivity of Wada et al.'s (78, 79) medium with only 0.15 % yeast extract suggest only catalytic and other low concentration functions are required from yeast extract for ethanol production. Hence, a study to replace the active components of yeast

Table 2.6

Growth Factor Requirements of *Saccharomyces cerevisiae*

Growth Factor	Number of Strains Requiring or Stimulated by Growth Factor		
Biotin	10	10	9
Pantothenic acid	10	12	9
myo-Inositol	4	5	8
Thiamine	1	2	8
Pyridoxine	0		4
Thiamine or pyridoxine		2	
p-aminobenzoic acid			1
Nicotinic acid		0	
Number of strains tested	10	12	9
References	Leonian and Lilly (41)	Schultz and Atkin (76)	Wiles (77)

Table 2.7

Growth Factor Requirements of Brewer's Yeast

(from Atkin et al (42))

Required Growth Factors	Number and Type of Strains Requiring Growth Factors (58 strains tested)
Biotin	14 Lager
Biotin, Pantothenic acid	23 Lager
Biotin, Inositol	4 Lager
Biotin, Pantothenic acid, Inositol	12 Lager
Biotin, Pantothenic acid, Inositol and either Thiamine or Pyridoxine	5 Ale

extract with growth factors appears feasible. Furthermore, Pirt (25) noted the need for a systematic study of vitamins as growth limiting nutrients. Solomons (62) and Weinshank and Garver (80) observed that pure vitamins and amino acids could be more economical than complex factors, such as yeast extract, in certain media.

There have been many other studies of the effect of individual nutrients or groups of nutrients on yeast growth and fermentation. However, most utilize complex or semi-complex media, most use batch cultures, and practically all use different yeast strains. Cysewski (81) studied the same strain used in this work, but with relatively high yeast extract concentration medium as shown in Table 1.1. Other relevant studies are referenced as they pertain to specific nutritional effects in later chapters.

2.3.2 Organism Analyses and Limitations

Minimum medium requirements for minerals and essential growth factors can be estimated in principle from the cell composition. Analyses from Harrison (82), White (17), Weinshank and Garver (80), and Eddy (35) are presented in Table 2.8. The variability of this data, particularly for the low concentration components, is a major limitation. These variations in cell composition are in large part the effects of different growth medium compositions. Another limitation to the application of cell composition data to medium design arises from the unknown concentration gradient required for transport of nutrients from the medium into the cell interior. As further discussed in Chapter 7, many of the major nutrients can be actively transported with saturation kinetics that have low saturation constants. However, transport kinetics for

Table 2.8

Yeast Composition

	Percentage of Yeast Dry Mass (Except Parts Per Million for Growth Factors)			
	Harrison (82)	White (17)	Eddy (35) (for 6 % ash)	Weinshank and Garver (80) Average (Range)
Protein				40 (35 - 45)
Carbohydrate				38 (30 - 45)
Lipid				8 (5 - 10)
Nucleic acid				8 (5 - 10)
C	45 - 47	47.0		48 (46 - 52)
H	6.0 - 6.5	6.0		
O	31 - 32	32.5		
N	7.5 - 9.0	8.5		7.5 (6 - 8.5)
K	0.9 - 3.5	2.1	1.7	
P	1.1 - 2.0	1.1	1.4	
S	0.3 - 0.5	0.01	0.046	
Mg	0.15 - 0.5	0.2	0.21	
Ca	0.04 - 0.9	0.04	0.15	
Na	0.02 - 0.2		0.038	
Zn	0.004 - 0.13			
Fe	0.003 - 0.1	0.005	0.13	
Cu	0.002 - 0.012	2.0×10^{-5}		
Mn	0.0004 - 0.0035			
Co	0.0005			
Mo	$5 - 9 \times 10^{-6}$			
Cl	0.004 - 0.1	0.02	0.030	
I	0.00005 - 0.0004			
Pb	0.0001 - 0.0007			
As	0.00001			
Al		0.003		
Si		0.04	0.028	
Total Ash		6.0		6 (4 - 10)
Growth Factors (ppm)				
biotin	0.6 - 2.0		0.5 - 1.8	
thiamine HCl	20 - 165		29 - 90	
pyridoxine HCl	20 - 50			
Ca-pantothenate	40 - 120		118 - 198	
riboflavin	45 - 100			
nicotinic acid	330 - 585		190 - 585	
folic acid	5 - 49		19 - 35	
p-aminobenzoic acid	5 - 160		8 - 95	
inositol	3000 - 5000		4320	
choline chloride	2710 - 5500			

many trace elements are not known.

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Chapter 3

3. Experimental Methods

This chapter will describe general procedures common to a number of experiments described in following chapters. Equipment and methods unique to individual experiments are described with those experiments.

3.1 Culture Maintenance

Saccharomyces cerevisiae (also known as Saccharomyces anamensis), American Type Culture Collection (ATCC) No. 4126, is the yeast strain used in this study. This organism was received as a freeze dried culture from ATCC, grown in YM broth (Difco 0711), and transferred to YM agar (Difco 0712) and nutrient agar (Difco 0634) slants. These slants were stored at about 4 ^{o}C. Transfers of cultures to new slants were made approximately every two months to maintain healthy yeast. However, after long continuous fermentations the yeast appeared to have changed by adaptation or mutation a number of times. If the changes were improvements, as in increased ethanol productivity or decreased medium requirements, the new culture would be saved and used in subsequent fermentations. Therefore, the present yeast should be considerably improved over the original yeast.

3.2 Medium Preparation

The specific procedures for medium preparation depended on the experiment. However, some general procedures can be described here. The major concerns in medium preparation are maintaining the integrity of the medium components during sterilization, reducing the probability of

contamination after sterilization, and reproducing given media compositions.

Maintaining the medium integrity requires prevention of reactions among and precipitation by the components. Thus, sterilization by filtration with 0.2 micron (micrometer) filters was used when feasible, namely, for small quantities of media or for media without complex components, such as yeast extract or cornsteep liquor. Otherwise, media sterilization was performed by autoclaving and by separate filtration of heat labile growth factors.

Even though yeast extract and cornsteep liquor contain heat labile components, large quantities for continuous cultures have to be autoclaved because they cannot be filtered though 0.2 micron openings sufficiently quickly. For smaller quantities of yeast extract, prefilters were used and changed often, but an insoluble residue always remained on the filter afterwards. Containing more suspended solids, cornsteep liquor is much more viscous and would leave even more residue. Thus, there is no apparent method of sterilization which completely maintains the integrity of the yeast extract or cornsteep liquor.

To minimize reactions among components during autoclaving, glucose was autoclaved separately from the minerals and yeast extract or cornsteep liquor, which were autoclaved together. The Maillard type browning reactions occur between the carbonyl groups of reducing sugars and the amino groups of amino acids and proteins. The amino acids are thereby inactivated (1). Moreover, the heating of glucose with phosphates at an alkaline pH has been reported to cause inhibition of some microorganisms although the cause is not understood (2).

To reduce the extent of these reactions, media were autoclaved at an acidic pH generally for about 20 minutes at 120 ^{o}C. Higher temperatures were used for larger quantities of media to reduce the heat-up time.

The major causes for precipitation during autoclaving are reactions between high concentrations of phosphates and the divalent cations, calcium and magnesium at alkaline pH. Thus, when this precipitation appeared likely, part or all of the phosphates were added after autoclaving with the growth factors by filtration.

Precipitation also occurred in the concentrated stock solutions of trace minerals after preparation. The cornsteep liquor also contains solids. Thus, these two suspensions had to be well mixed before being added to the media. Furthermore, the final media had to be continuously stirred if solids remained during continuous feeding to the fermentor.

To reduce the probability of bacterial contamination of the media during continuous fermentations, the following antibiotics were usually added to the feed media based on the composition used by Perry, Beezer, and Miles (3):

Penicillin-G, potassium salt	1000 units/ml, 1585 units/mg
Ampicillin	0.1 mg/ml
Streptomycin sulfate	0.02 mg/ml

Controlled continuous cultures showed these antibiotics had no significant effects on yeast fermentations. To inhibit fungal contamination, the feed media was sparged with nitrogen to reduce the oxygen required for most fungal growth. Contamination by a Penicillum resembling fungus was especially common and troublesome.

Feed media were normally prepared from concentrated stock solutions of most individual components to make media formulations as reproducible as possible. Except as noted, J.T. Baker reagent grade glucose, Difco certified yeast extract, Corn Products Unit of CPC International Argo Steepwater E801 cornsteep liquor, reagent grade minerals and vitamins were used in the feed media. The media components except for the yeast extract and cornsteep liquor should have a consistent quality. However, the yeast extract consistency is questionable because its color appeared variable. The cornsteep liquor did not come sterile and was not kept sterile although growth was not observed during refrigerated storage. Nevertheless, nutrients could have been consumed.

The other main source of inconsistency in some media preparation was from the different time temperature histories of autoclaving different volumes of even the same composition media. The volumes were maintained as consistent as possible to avoid this effect. This effect would be especially important to avoid if reactions occurred among components during autoclaving.

3.3 Assay Methods

3.3.1 Sugar Analysis

3.3.1.1 Reducing Sugar Analysis

Reducing sugars were measured by the Dinitrosalicylic Acid (DNS) Method developed by Summer (4) and modified by Sciamanna (5). This method is capable of measuring approximately 0.5 to 2.5 g/L reducing sugars with a linear optical density response to concentration at 600 nm. Higher reducing sugar concentrations must be reduced to this range

by dilution. For concentrations above 1 g/L, the DNS method results are not significantly different from the Glucose Oxidase Peroxidase results for measurement of glucose (described below).

3.3.1.2 Glucose Analysis

Glucose was originally analyzed by the Glucose Oxidase Peroxidase Method, which is based on the following enzymatic reactions.

Glucose oxidase mediated reaction:

$$\text{Glucose} + O_2 + H_2O = H_2O_2 + \text{gluconic acid}$$

Peroxidase mediated reaction:

$$H_2O_2 + \text{reduced chromogen} = \text{oxidized chromogen} + H_2O$$

The concentration of the oxidized chromogen is determined spectrophotometrically at 436 nm.

As modified by Long (6), this method can be used in the range 0.1-2.0 g/L glucose and is more specific to glucose than the DNS method. The drawbacks of this method are the long incubation time (one hour at 37 ^{o}C.) and the use of the known carcinogen o-dianisidine for the reduced chromogen. A variation of this method is given in the Worthington Enzyme Manual (7).

For later analyses, glucose was measured with the Instrumentation Laboratory Model 919 Glucose Analyzer, which also utilizes glucose oxidase and peroxidase enzymatic reactions, but without the drawbacks of the earlier method. The lower limit of accuracy for the Glucose Analyzer was similarly about 0.1 g/L glucose, but its upper linear limit was reported at about 6.0 g/L. The automatic sampling facility of this machine further made this the method of choice for glucose analysis.

3.3.2 Ethanol Analysis

3.3.2.1 Enzymatic Analysis

The enzymatic determination of ethanol is based on the following reaction mediated by crystalline horse liver alcohol dehydrogenase:

$$\text{Ethanol} + NAD^+ = \text{Acetaldehyde} + NADH + H^+$$

This reaction is followed spectrophotometrically by measuring the absorbance of NADH at 340 nm.

This method as proposed by Theorell and Bonnichsen (8) can measure 0.07 to 1.4 micromoles ethanol, but is not very linear even in this low range. Thus, samples require much dilution. This method, however, is useful for large numbers of low ethanol concentration samples for which gas chromatography is not appropriate. A variation of this method is also in the Worthington Enzyme Manual (7).

3.3.2.2 Gas Chromatography Analysis

Practically all the ethanol analyses were done by gas chromatography using the Varian Aerograph 1520. The column is six feet by 1/4 inch O.D. and has been packed with Porapak Q and Chromosorb 101, 100-120 mesh. The column oven temperature ranged from 160 to 225 ^{o}C. The flame ionization detector temperature and injector temperature were 230 and 250 ^{o}C., respectively.

Initially, peak areas were determined by multiplying the peak heights by the width at one half of the maximum height level or by using a mechanical disc chart integrator. For later analyses, a Varian CDS 111 digital integrator was used. In all cases calibration curves were

used to determine sample ethanol concentrations, normally with less than 5% error.

3.3.3 Cell Mass Analysis

Rapid estimates of dry cell mass were obtained by measuring the cell suspension optical density (O.D.) at 600 nm with either the Bausch and Lomb Spectronic System 400 or the Beckman DU-2 Spectrophotometer. Calibrations, such as that shown in Figure 3.1, were used to convert optical density readings to dry cell mass. The linear calibration range is approximately from 0 to 0.5 optical density units; the points above this range were determined by diluting to this range and multiplying the results by the dilution factor.

Optical densities provide a convenient method of monitoring the growth rate of batch cultures and of following the approach to steady state for continuous cultures. However, optical density is not solely a function of cell mass, but also of cell morphology, size, and number, all of which affect the cell surface area and, hence, the scattering of light. For example, cell size has been shown to be a function of the growth phase and thus of the growth rate (9). Figure 3.2 derived from steady state continuous cultures show the effect of specific growth rate on the ratio of dry cell mass to cell O.D. Assuming the cell O.D. is proportional to the surface area of the total cell mass, the increasing trend of this ratio with respect to specific growth rate implies the cell diameter is increasing up to about 0.313/hr.

Therefore, direct dry weight measurements were made to support the O.D. readings as well as to provide calibration points for them. In

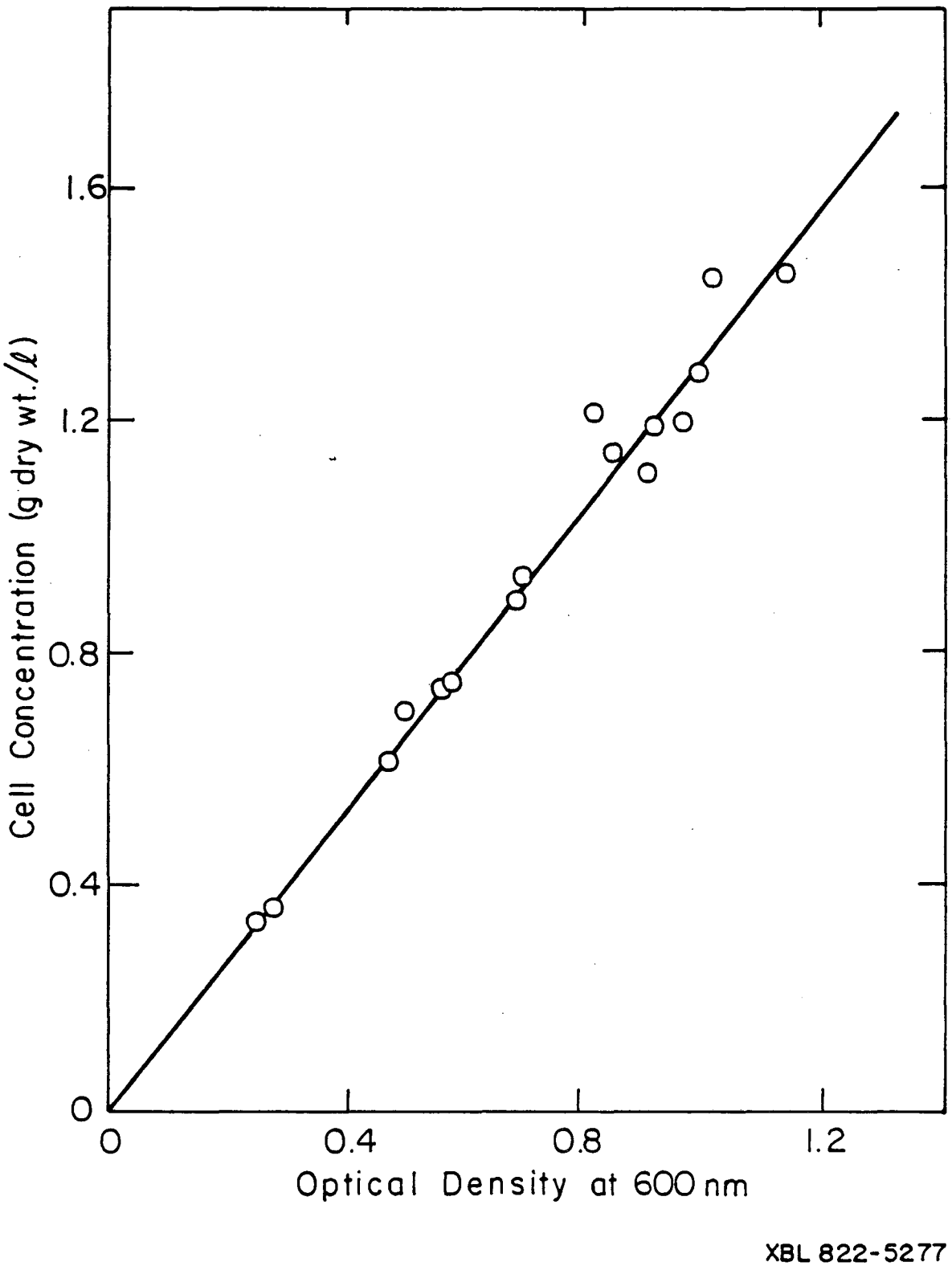

Figure 3.1 Dry Cell Mass Calibration for Saccharomyces cerevisiae

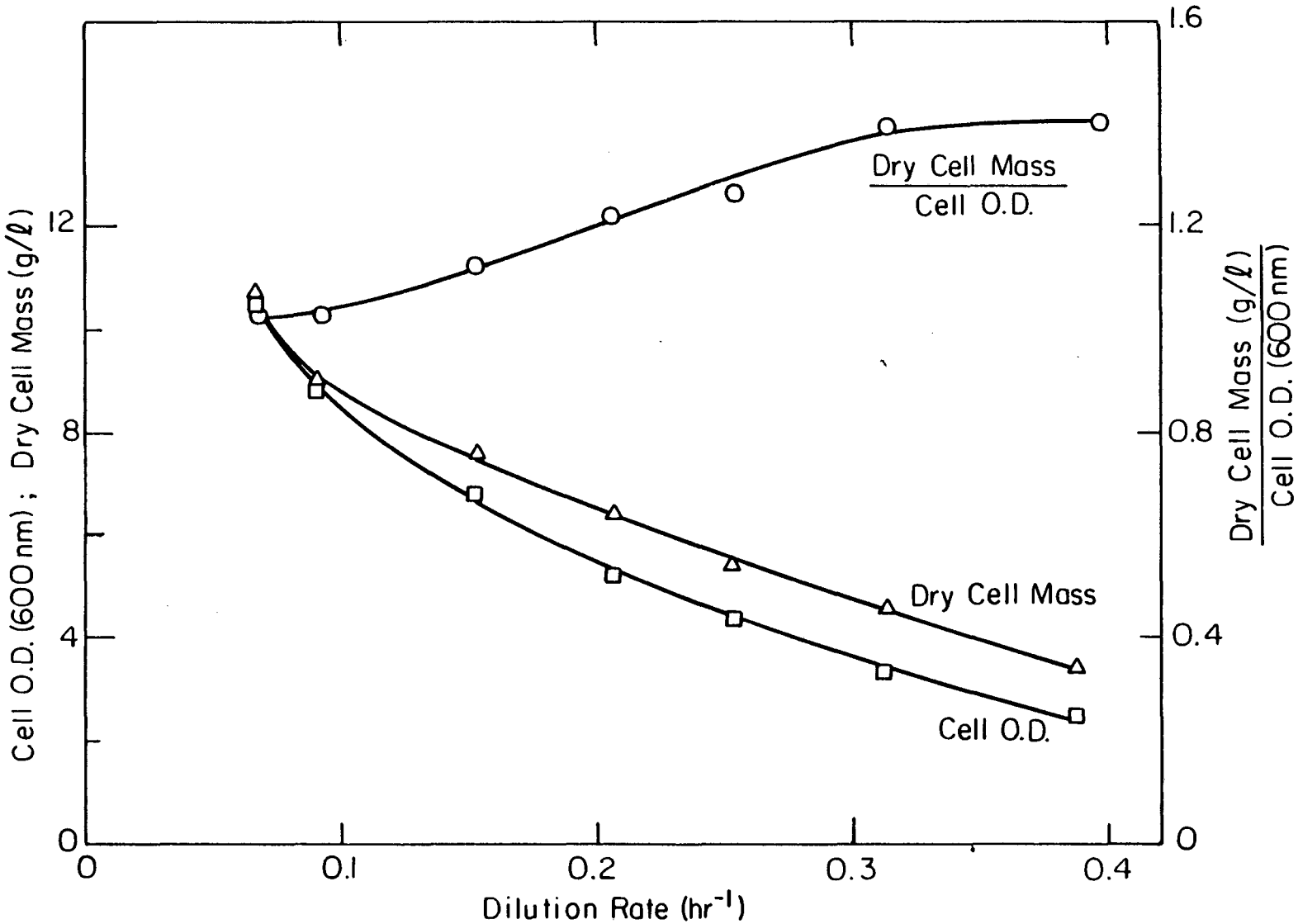

Figure 3.2 Effect of Dilution Rate on the Ratio of Dry Cell Mass to Optical Density at 600 nm for Saccharomyces cerevisiae

particular, dry weight measurements were taken for all continuous culture steady states, while O.D. readings were taken to help determine when steady states were reached. The dry weights were measured by vacuum filtration of 10 to 30 ml cell suspension, depending on its concentration, through pre-weighed 37 mm Nuclepore filters with 0.4 micron pores. Approximately 10 ml distilled water was then used to wash undissolved medium components from the cells. The cells and filter were then dried in a 70 ^{o}C. oven for at least a week to reach a constant weight. After cooling in a desiccator, the weight was measured on a microanalytical balance accurate to 0.1 milligram. However, larger errors probably occur during filtration such that the overall error in dry cell mass can be about 5-10%.

3.3.4 Yeast Viability

A major shortcoming of both the optical density and filtration methods of cell mass determination is that live and dead cells are not distinguished. To make this distinction the cells were stained with a 2 g/L methylene blue solution with approximately equal volumes of cell suspension and staining solution. After five minutes exposure, counts can be made of dead cells which are deeply stained and viable cells which are not stained as determined through the oil immersion lens of a Reichertz "Zetopan" Research microscope. A Polaroid camera was attached to this microscope to record the observations.

3.3.5 Gas Analysis

The inlet to and the outlet gas from continuous fermentations were analyzed for oxygen, nitrogen, and carbon dioxide. These analyses

provided an independent measure of fermentation activity from the carbon dioxide evolution rate, allowed a carbon mass balance to be made, and gave a rough estimate of the oxygen tension and consumption.

As shown in Figure 3.3, a Varian Aerograph A90-P3 gas chromatograph set up with two columns in series performed the gas analyses. The first column (6 ft x 1/4 inch O.D.) packed with Porapak Q separates carbon dioxide from the mixture of oxygen, nitrogen and argon. The second column (20 ft x 1/4 inch O.D.) packed with molecular sieve 13X, 30/60 mesh, separates the oxygen and argon from the nitrogen. Although the oxygen and argon are not separated, their ratio can be estimated. Further information on this method is given by Thompson (10).

The Porapak Q column was in an oven controlled at 125 ^{o}C.; the molecular sieve column attached outside the chromatograph was at room temperature. Temperatures of the injector, collector and detector were 245, 195, and 230 ^{o}C., respectively. The filament current for the thermal conductivity detector was set at 180 ma. Helium was used as the carrier gas at a flow of about 40 ml/min.

Gas samples were taken with gas tight syringes in volumes ranging from 25 to 500 microliters. All the gases analyzed have linear responses at least up to 500 microliters, which was the normal volume used. The two types of syringes used were the Hamilton Gastight Syringe #1750 and the Scientific Glass Engineering Gas Tight Syringe 500A-RN-GSG. A separate calibration was made for each gas either from precalibrated gases or from mixing varying volumes of air, pure nitrogen, and pure carbon dioxide. Except for very low concentrations, the error should be less than 5% for each gas concentration.

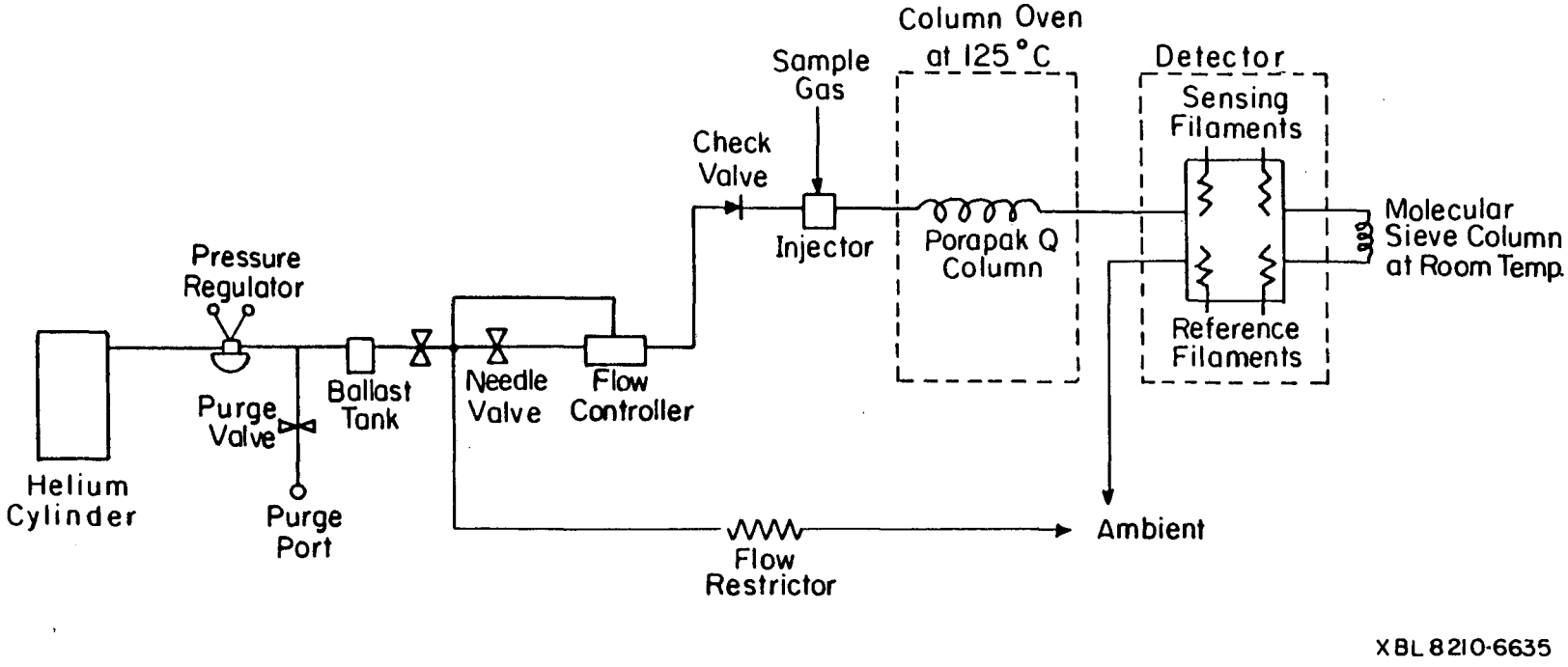

Figure 3.3 Gas Chromatograph Setup for Analyses of O_2, N_2, and CO_2

3.4 References for Chapter 3

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Chapter 4

4. Selection of the Ethanol Producing Microorganism

4.1 Microorganisms Evaluated

Detailed criteria for selecting the optimum organism for ethanol production are given in section 1.2 of the Introduction. An exhaustive search for all organisms which may meet these criteria is beyond the scope of this work. Instead the search has been limited to the following organisms, which are available from the American Type Culture Collection (ATCC) (1).

(1) Saccharomyces cerevisiae, ATCC 4126

Also known as Saccharomyces anamensis, this strain is reported to ferment at high temperatures and has been used in industrial ethanol production in the Amylo process (1,2). It has also been used in laboratory research for studies of the Rotorfermentor by Margaritis (3), ethanol inhibition by Bazua (4) and Hoppe and Hansford (12), hollow-fiber membrane fermentors by Vick Roy (13) and Inloes (14), urea inhibition by Dove (5), and vacuum fermentation processes by Cysewski (2) and Maiorella (6).

(2) Saccharomyces cerevisiae, ATCC 4132

This strain is a molasses distillery yeast with a high temperature optimum (1).

(3) Saccharomyces species, ATCC 764

This unidentified species is a Hungarian beer yeast, ferments waste sulfite liquor, and is acid tolerant (1).

(4) Candida utilis, ATCC 9226

This is a food yeast, also known as a torula yeast. It has the advantage over Saccharomyces yeasts of being able to utilize xylose as well as glucose.

(5) Torula thermophile, ATCC 16463

As its name implies, this is a relatively thermophilic yeast with a recommended preservation and maintenance temperature of 40 ^{o}C. It was isolated from chicken nest straw (1,7).

(6) Mucor pusillus, ATCC 24923

This fungus was isolated from barley kernel and able to degrade barley arabinoxylan and carboxymethyl cellulose with a temperature range of 24-37 ^{o}C. (1,8).

(7) Rhizopus formosaensis, ATCC 26612

Isolated from the soil with a temperature range of 30-40 ^{o}C., this fungus has been used in the Amylo process for high alcohol productivity (1,9).

The above organisms have the common feature of relatively high temperature optima for ethanol producers. High temperature tolerant organisms are important for processes, such as vacu-ferm and flashferm, where the organisms are subjected to vacuum conditions. Higher temperatures allow higher pressures for evaporation of ethanol. Higher pressures greatly reduce vacuum compressor requirements, which dominate the capital equipment costs of ethanol production (10). High temperature fermentations also lower cooling costs since the fermentations are

exothermic (11,15,16).

Furthermore, as yeasts and fungi, all the above organisms are easily separated for recycle operations, which allow high cell mass concentrations in the fermentor. For laboratory research, yeasts are easier to work with because they are easier to keep well mixed in a fermentor and because they are less likely to grow on walls and in feed lines. As facultative anaerobes with tolerance to low pH, yeasts are also less likely to become contaminated. All the organisms are nonpathogenic. The Saccharomyces and Candida yeasts have been used industrially.

In this study selection is made for organisms which are thermophilic, high ethanol yielding, not slow ethanol producing, and non fastidious in nutritional requirements. Ethanol tolerance and stability to adaptation and mutation evaluations require long term continuous culture studies beyond the scope of this work for more than one organism.

4.2 Method of Microorganism Evaluation

The above seven organisms were evaluated for their relative abilities to ferment glucose to ethanol in 35 ml test tubes filled to about 20 ml and incubated at 30, 35, and 40 ^{o}C. The composition of the semisynthetic medium containing 20 g/L glucose initially is given in Table 4.1. This composition selects for organisms with relatively nonfastidious and readily available feed components. The yeast extract provides the growth factors and the undefined complex factors which should support the growth of most yeast and other fungus. To be economical, however, less expensive substitutes need to be found for the active

Table 4.1

Medium for Organism Evaluation

Component	Concentration (g/L)
Glucose (J.T.Baker reagent grade)	20.0
$(NH_4)_2SO_4$	2.06
KH_2PO_4	2.0
Yeast Extract (Difco certified)	0.5
$CaCl_2 \cdot 2H_2O$	0.4
$MgSO_4 \cdot 7H_2O$	0.3
H_3BO_3	0.01
$ZnSO_4 \cdot 7H_2O$	0.01
$CuSO_4 \cdot 5H_2O$	0.004
$MnSO_4 \cdot H_2O$	0.003
$Al_2(SO_4)_3$	0.003
$FeSO_4 \cdot 7H_2O$	0.002
$CoSO_4 \cdot 7H_2O$	0.001
KI	0.001

components of yeast extract. Batch cultures with relatively low initial substrate concentrations allow the organisms to grow initially at their maximum rates without substrate limitations and with minimal by-product inhibition.

Because only qualitative comparisons of the organisms at different temperatures were expected, temperature was the only controlled variable. The ethanol and cell mass produced and the residual glucose were measured after one and two days. Glucose was measured using glucose oxidase and peroxidase, ethanol using alcohol dehydrogenase and NAD^+, and dry cell mass by filtration. These measurement techniques are described in the Analytical Measurements section of Chapter 3.

4.3 Results and Discussion

The results of the batch yeast fermentations are given in Table 4.2. The results for the two fungi, Mucor pusillus and Rhizopus formosaensis, were incomplete and only the residual glucose data are reported. Available cell mass yields (g-dry cell/ g-glucose consumed) and ethanol yields (g-ethanol/ g-glucose consumed) are reported in Table 4.3. Because the substrate concentrations are quite low, the dry cell mass, ethanol and residual glucose concentrations are also low. Thus, the values reported in Table 4.2 are subject to relatively high percentage measurement errors. The ethanol measurement errors are compounded by the evaporation losses of ethanol as noted by the decrease in ethanol from the first to the second day in many cases. Also in the absence of glucose the ethanol may be utilized oxidatively as a carbon source by the cells. These errors are then propagated in the calculations of cell mass yields and ethanol yields in Table 4.3. Where one and two day

Table 4.2

Batch Fermentation of 20 g/L Glucose by Thermophilic Organisms

			30 °C.			35 °C.			40 °C.		
ATCC #	Organism	Time (days)	Dry Cell Mass (g/L)	Ethanol (g/L)	Residual Glucose (g/L)	Dry Cell Mass (g/L)	Ethanol (g/L)	Residual Glucose (g/L)	Dry Cell Mass (g/L)	Ethanol (g/L)	Residual Glucose (g/L)
4126	Saccharomyces cerevisiae	1	1.2	6.9		1.2	7.7	0.11	0.70	6.7	2.7
		2	1.3	5.1	0.007	1.1	8.2	0.021	0.76	6.6	0.024
4132	Saccharomyces cerevisiae	1	0.80	8.2	0.015	0.84	6.2	0.052	0.28	0.94	17.
		2	0.94	7.0	0.006	0.96	6.5	0.01	0.035	0.17	20.
764	Saccharomyces species	1	0.84	8.1	0.020	0.74	8.1	0.014	0.36	3.0	12.
		2	0.90	7.1	0.007	0.60	8.6	0.01	0.20	1.1	9.3
9226	Candida utilis	1	0.38	2.0	14.	0.64	4.7	8.3	0.38	2.5	13.
		2	0.56	3.5	11.	0.68	5.8	5.6	0.50	2.2	8.3
16463	Torula thermophile	1	0.005	0.0	20.	0.015	0.0	20.	0.035	0.0	20.
		2	0.005	0.0	14.	0.025	0.0	17.	0.015	0.0	16.
24923	Mucor pusillus				15.			13.			13.
26612	Rhizopus formosaensis				9.7			5.2			4.8

Table 4.3

Cell Mass and Ethanol Yield Coefficients[1]

		30 °C.		35 °C.		40 °C.	
ATCC #	Organism	Cell Mass Yield	Ethanol Yield	Cell Mass Yield	Ethanol Yield	Cell Mass Yield	Ethanol Yield
4126	Saccharomyces cerevisiae	0.065	0.35	0.061	0.41	0.041	0.39
4132	Saccharomyces cerevisiae	0.047	0.41	0.048	0.33	0.11	0.36
764	Saccharomyces species	0.045	0.41	0.037	0.43	0.044	0.38
9226	Candida utilis	0.063	0.39	0.055	0.40	0.054	0.35
16463	Torula thermophile		0.0		0.0		0.0

[1]Cell Mass Yield units: g-dry cell mass/g-glucose consumed

Ethanol Yield units: g-ethanol/g-glucose consumed

yields differ, the higher values are selected.

It should be noted that no pH or dissolved oxygen control was used in these fermentations. Thus, the glucose consumption rates and ethanol yields should be improved for all organisms if the pH and dissolved oxygen were optimized. Thus, only qualitative comparisons should be made between organisms based on Tables 4.2 and 4.3.

Glucose consumption rates, ethanol yields, and temperature optima are the most important criteria in this evaluation of organisms. Only the Saccharomyces yeasts were able to essentially fully utilize the glucose after one or two days. ATCC strains 4132 and 764 could fully utilize the glucose only at 30 and 35 ^{o}C., but strain 4126 could also fully utilize the glucose at 40 ^{o}C. The maximum ethanol yields (g-ethanol/g-glucose consumed) for strains 4126, 4132, and 764 were 0.41 at 35 ^{o}C., 0.41 at 30 ^{o}C., and 0.43 at 35 ^{o}C., respectively. These ethanol yields are not significantly different with respect to the experimental error. Because strain 4126 is more thermotolerant and has been extensively studied, it was the organism selected.

4.4 Conclusions

(1) The three Saccharomyces strains are comparable ethanol producers in the range 30-35 ^{o}C.

(2) Saccharomyces cerevisiae ATCC 4126 is the highest ethanol producer of all organisms tested at 40 ^{o}C.

(3) Candida utilis can not utilize glucose as rapidly as the Saccharomyces yeasts at 30 and 35 ^{o}C. but can utilize glucose as well as

Saccharomyces cerevisiae 4132 and Saccharomyces species 764 at 40 ^{o}C.

(4) The cell and ethanol yields for Candida utilis are comparable to those of Saccharomyces yeasts.

(5) Torula thermophile cannot ferment glucose to ethanol under the given conditions.

(6) Saccharomyces cerevisiae ATCC 4126 is the organism selected from this study because of its extensive use in industry and in research and because of its relatively high temperature optimum.

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Chapter 5

5. Identification of Growth Factors

5.1 Introduction

As discussed in Chapter 2, expensive complex components are often added to media to provide unknown required growth factors. These growth factors may be either essential or stimulatory to cell growth and ethanol production rates. Because growth factor requirements differ among strains of the same species, they must be experimentally determined for individual strains. In this chapter the component requirements for Saccharomyces cerevisiae ATCC No. 4126 are identified, and their concentration bounds are estimated in batch cultures. Furthermore, the feasibility of replacing high yeast extract concentrations with these identified growth factors in purely synthetic media is shown. With approximate bounds established from batch cultures, these component requirements will be quantitatively determined in continuous cultures under various conditions in later chapters.

The seven growth factors tested for this strain were those required by other strains of the same species as reported in Chapter 2. For comparison, yeast extract was also tested. First, a preliminary study of the effects of the growth factors and yeast extract on cell mass yield and ethanol yield was carried out in shake flask screenings. Second, the effect of important growth factors, yeast extract, and certain amino acids on growth and ethanol production rates was studied. Initially this study was also in shake flasks, but subsequently in better controlled one liter batch Miniferm fermentors from New Brunswick Scientific Co.,

Inc. Third, additional results on the effect of growth factors on cell mass and ethanol yields were obtained concurrently with the rate studies but are presented after the rate data.

5.2 Preliminary Study of the Growth Factor Effects on Cell Mass and Ethanol Yields

5.2.1 Growth Factors Studied

Fifteen shake flask cultures with different combinations of seven growth factors and yeast extract were run to determine their effects on cell and ethanol yields. The compositions of these 15 media are given in Tables 5.1 and 5.2. Most of the growth factors commonly required by Saccharomyces cerevisiae are present in the synthetic mix of Medium 8. The effect of individual growth factor deficiencies, their complete deficiency, their supplementation with yeast extract, and other combinations of growth factors are to be determined from these media. The initial glucose concentration was set at about 50 g/L to prevent ethanol inhibition from significantly masking nutrient limitation effects. The bases for the common base medium is discussed in Chapter 2.

5.2.2 Procedure for Cell Mass and Ethanol Yields

All media were sterilized with 0.2 micron filters. The 250 ml shake flasks were filled to about 50 ml and incubated at 35 ^{o}C. with 200 rpm agitation. Shake Flask Set 1 (original cultures) was inoculated each with one loop of inoculum grown on the basal medium plus 1.0 g/L yeast extract. After five days of batch fermentation, samples were taken for ethanol, residual glucose and cell dry weight determination. Then 0.1 ml of each shake flask was used to inoculate Shake Flask Set 2 (first culture transfers) filled to 50 ml with the same original media

Table 5.1

Base Medium for Cell Growth and Ethanol Production Yield and Rate Studies Used in Shake Flask Sets 1-5

Component	Concentration (g/L)
Glucose (J.T. Baker reagent grade)	50.0
$(NH_4)_2SO_4$	5.19
KH_2PO_4	1.53
$MgSO_4 \cdot 7H_2O$	0.55
$CaCl_2 \cdot 2H_2O$	0.13
H_3BO_3	0.01
$ZnSO_4 \cdot 7H_2O$	0.01
$CuSO_4 \cdot 5H_2O$	0.004
$MnSO_4 \cdot H_2O$	0.003
$Al_2(SO_4)_3$	0.003
$FeSO_4 \cdot 7H_2O$	0.002
$CoSO_4 \cdot 7H_2O$	0.001
KI	0.001

Table 5.2

Growth Factors for Cell and Ethanol Yield Studies in Shake Flask Sets 1-3

Growth Factors	Media (mg/L)							
	1	2	3	4	5	6	7	8
d-biotin		0.125	0.125	0.125	0.125	0.125	0.125	0.125
Ca d-pantothenate	6.25		6.25	6.25	6.25	6.25	6.25	6.25
myo-inositol	125.	125.		125.	125.	125.	125.	125.
thiamine HCl	5.0	5.0	5.0		5.0	5.0	5.0	5.0
pyridoxide HCl	6.25	6.25	6.25	6.25		6.25	6.25	6.25
Na p-aminobenzoate	1.0	1.0	1.0	1.0	1.0		1.0	1.0
nicotinic acid	5.0	5.0	5.0	5.0	5.0	5.0		5.0
yeast extract								

Growth Factors	Media (mg/L)						
	9	10	11	12	13	14	15
d-biotin	0.125				0.125	0.125	
Ca d-pantothenate	6.25				6.25	6.25	
myo-inositol	125.				125.		
thiamine HCl	5.0				5.0		
pyridoxine HCl	6.25						
Na p-aminobenzoate	1.0						
nicotinic acid	5.0						
yeast extract	500.	500.	1500.				

compositions. This second set of cultures was incubated for three days, sampled, and then used to inoculate Shake Flask Set 3 (second culture transfers) filled to 50 ml with the same media with an inoculum volume of 0.01 ml. This third set was incubated for three days and then sampled. The transfers of cells between cultures as inocula reduced the nutrients carried over from the original inoculum, which was only a loop (approximately 0.001 ml). Dry weights of cells were determined by filtration, ethanol concentrations by gas chromatography, and residual glucose concentrations by DNS (See Chapter 3).

5.2.3 Results for Cell Mass and Ethanol Yields

The effects of growth factors on cell and ethanol production and on glucose consumption are given in Table 5.3. The corresponding cell mass yields and ethanol yields are reported in Table 5.4. As shown by Media 12 and 15, practically no cell growth or ethanol production could occur within 3 to 5 days of inoculation without explicit addition of growth factors or yeast extract. Comparing the other media with growth factors, the deficiency of biotin (Medium 1) has the greatest effect by at least a factor of two on reduced cell and ethanol production and especially glucose consumption. Without biotin, only 17-30 g/L glucose is consumed out of approximately 50 g/L. Without any of the other six growth factors, less than 2.0 g/L maximum is unconsumed out of about 50 g/L. The biotin deficient medium also has the lowest cell mass yield, but its ethanol yield is comparable to many other growth factor deficient media.

Deficiencies of growth factors other than biotin (Media 2 - 7) also reduce cell yield but to less extent. The complete synthetic growth

Table 5.3

Effects of Growth Factors on Cell Mass, Residual Glucose, and Ethanol

Media	Dry Cell Mass(g/L)			Residual Glucose (g/L)			Ethanol (g/L)		
	Shake Flask Set			Shake Flask Set			Shake Flask Set		
	1	2	3	1	2	3	1	2	3
	Original	Transfer #1	Transfer #2	Original	Transfer #1	Transfer #2	Original	Transfer #1	Transfer #2
Base				52.	53.	54.			
1	0.20	0.92	0.57	22.	29.	37.	8.3	6.3	5.0
2	2.4	1.9	2.4	0.83	0.18	0.35	15.	20.	18.
3	2.7	2.6	2.7	1.4	0.64	0.020	15.	16.	20.
4	3.1	3.1	2.2	1.0	0.22	0.49	12.	16.	18.
5	2.5	2.6	2.1	1.2	0.31	0.0	12.	17.	21.
6	3.0	2.6	2.0	1.4	0.36	0.11	11.	14.	23.
7	3.3	2.5	2.7	0.29	0.22	0.11	12.	17.	20.
8	3.3	3.3	2.2	0.54	0.48	0.00	10.	13.	22.
9	3.6	2.6	2.8	0.71	0.22	0.12	9.6	16.	15.
10	3.4	2.7	2.9	1.4	0.31	1.5	11.	17.	16.
11	3.9	4.2	4.1	0.43	0.16	0.64	12.	17.	16.
12	0.14	0.026	0.11	50.	54.	51.	0.40	0	0
13	3.1	2.6	2.8	0.93	0.11	0.30	10.	18.	19.
14	3.3	2.8	3.2	1.2	0.53	0.81	12.	16.	16.
15	0.24	0.13	0.11	54.	52.	52.	0.20	0.66	0

Table 5.4

Effects of Growth Factors on Cell Mass Yield and Ethanol Yield

Media	Cell Mass Yield (g-dry cells/g-glucose consumed)				Ethanol Yield (g-ethanol/g-glucose consumed)			
	Shake Flask Sets				Shake Flask Sets			
	1 Original	2 Transfer #1	3 Transfer #2	Average	1 Original	2 Transfer #1	3 Transfer #2	Average
1	0.030	0.039	0.033	0.034	0.27	0.26	0.29	0.28
2	0.048	0.046	0.045	0.046	0.29	0.38	0.35	0.34
3	0.053	0.049	0.051	0.051	0.29	0.30	0.37	0.32
4	0.062	0.059	0.040	0.054	0.24	0.31	0.35	0.31
5	0.050	0.049	0.039	0.046	0.23	0.32	0.38	0.31
6	0.060	0.050	0.037	0.049	0.22	0.27	0.42	0.31
7	0.064	0.047	0.050	0.053	0.23	0.32	0.37	0.31
8	0.064	0.063	0.040	0.056	0.20	0.25	0.42	0.29
9	0.070	0.049	0.052	0.057	0.19	0.30	0.29	0.26
10	0.067	0.051	0.056	0.058	0.22	0.32	0.30	0.28
11	0.077	0.079	0.078	0.078	0.24	0.30	0.31	0.28
12								
13	0.061	0.048	0.053	0.054	0.20	0.34	0.35	0.30
14	0.066	0.054	0.061	0.060	0.23	0.30	0.30	0.28
15								

factor mixture (Medium 8) appears equivalent to 0.5 g/L yeast extract (Medium 10) with respect to cell yield. Base medium supplemented with only 1.5 g/L yeast extract (Medium 11) achieved the highest cell yield.

The relative effects of various growth factor deficiencies on ethanol yields appear less than on cell yields. Generally, ethanol yields are inversely proportional to cell yield as indirectly indicated in Figure 5.1. This figure shows the trade-off between ethanol yield $Y_{p/x}$ and cell mass concentration X as a function of growth factors and yeast extract. The product of $Y_{p/x}$ and X is the ethanol concentration, which is maximum for the pantothenate deficient Medium 2. The highest ethanol yielding media are those deficient in growth factors whereas the media yielding highest cell mass are those with complete growth factors and/or yeast extract. The yeast extract is expected to produce more cell mass because it contains amino acids, growth factors, and readily assimilated precursors. Conversely, growth factor deficiencies may limit cell growth but not ethanol production unassociated with growth.

Although the data in Tables 5.3 and 5.4 show scatter for transfers of certain media, using the average results from three shake flasks for each medium should reduce the random experimental error. The lack of control on pH, dissolved oxygen and ethanol loss should affect the relative results among the media studied less than the absolute results.

5.3 Growth Factor Effects on Cell Growth and Ethanol Production Rates

The effects of growth factors on cell mass growth and ethanol production rates in batch fermentation were studied in shake flasks and in Miniferm fermentors. The shake flasks provided initial screening of the

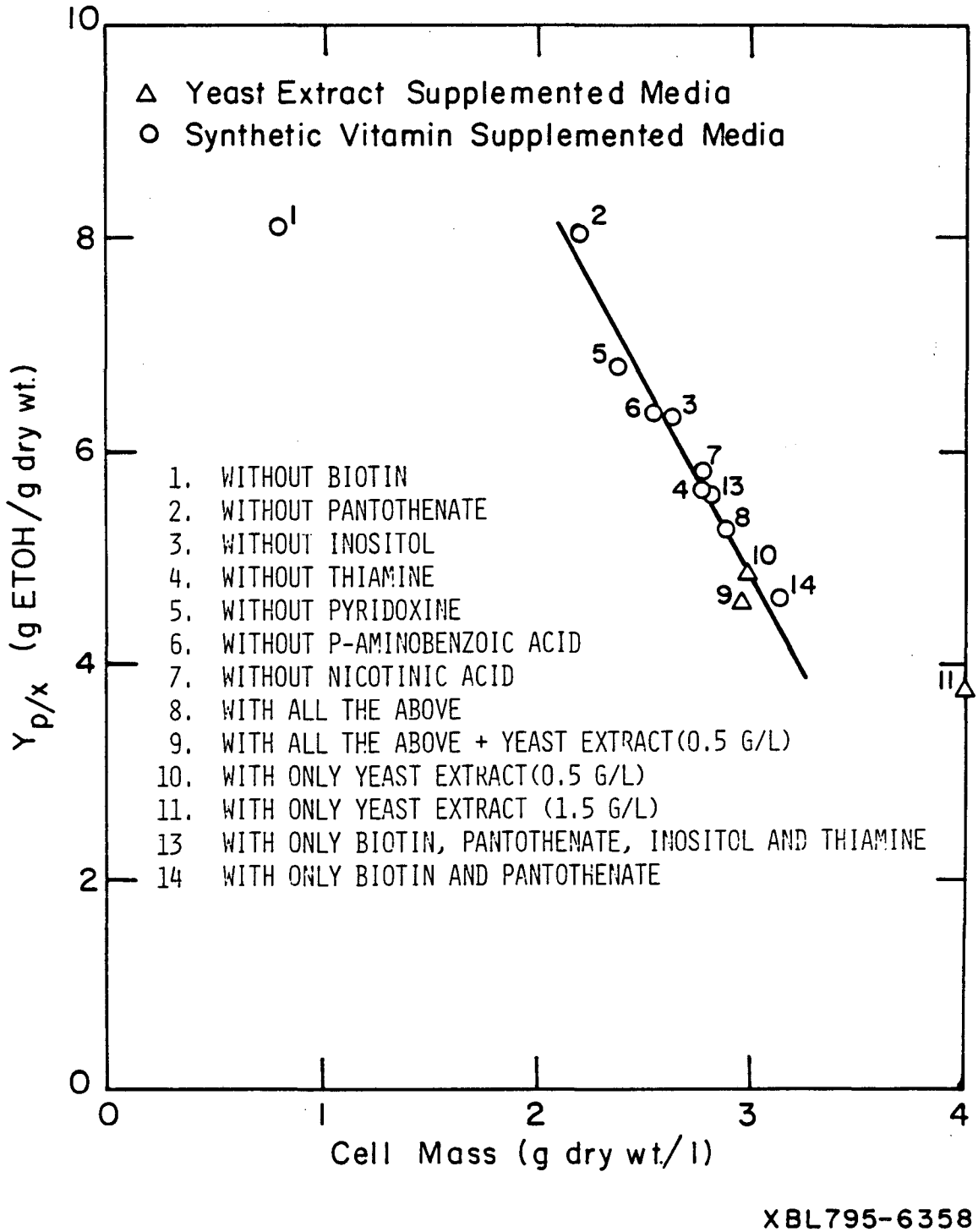

Figure 5.1 Effect of Growth Factors on Ethanol Yield $Y_{p/x}$ versus Cell Mass Produced in Shake Flask Cultures

effects of various growth factor combinations and of the effects of autoclaving the growth factors. Miniferm fermentors were then used for more precise kinetic studies on the effects of synthetic growth factors, yeast extract, and various amino acids.

The major parameters desired from the shake flask and Miniferm studies are the maximum specific growth rate (1/hr) and the maximum specific ethanol productivity (g-ethanol/g-cell/hr). These rates were determined during the exponential growth periods when all media components are still in excess.

5.3.1 Shake Flask Studies

5.3.1.1 Media Studied

The base medium in Table 5.1 for all the shake flask studies of growth factor effects on rates is the same as that used for the cell and ethanol yield studies. Table 5.5 presents the various growth factor combinations studied for rate effects. The effects of individual growth factor deficiencies are again studied with respect to the four most commonly required growth factors (Medium 4-5), the expanded set of seven possibly required growth factors (Media 4-6 and 5-3), and yeast extract. Furthermore, the effects of autoclaving yeast extract and synthetic growth factors (Media 5-6 and 5-7) and filtration of these components (Media 5-4 and 5-3) will be compared.

5.3.1.2 Procedure

Shake Flask Set 4 were inoculated from the corresponding composition culture in Shake Flask Set 3 except Medium 4-8 was inoculated from

Table 5.5

Growth Factors for Shake Flask Studies of Cell Growth and Ethanol Production Rates

Growth Factors	Media (mg/L)							
	4-1	4-2	4-3	4-4	4-5	4-6	4-7	4-8
d-biotin		0.50	0.50	0.50	0.50	0.50		
Ca d-pantothenate	6.25		6.25	6.25	6.25	6.25		
myo-inositol	125.	125.		125.	125.	125.		
thiamine HCl	5.0	5.0	5.0		5.0	5.0		
pyridoxine HCl						6.25		
Na p-aminobenzoate						1.0		
nicotinic acid						5.0		
yeast extract							500.	

Growth Factors	Media (mg/L)							
						autoclaved		
	5-1	5-2	5-3	5-4	5-5	5-6	5-7	5-8
d-biotin		0.50	0.50				0.50	
Ca d-pantothenate	6.25		6.25				6.25	
myo-inositol	125.		125.				125.	
thiamine HCl	5.0		5.0				5.0	
pyridoxine HCl	6.25		6.25				6.25	
Na p-aminobenzoate	1.0		1.0				1.0	
nicotinic acid	5.0		5.0				5.0	
yeast extract				500.	200.	500.		

the Medium 3-13 culture of Shake Flask Set 3. Therefore, there should be insignificant carry over with the inocula of the growth factors intended to be deficient. However, Shake Flask Set 5 were inoculated from a common culture grown on a medium containing 2 g/L yeast extract, centrifuged, and washed before final transfer to Shake Flask Set 5. Except as noted in Table 5.5, the growth factors and yeast extract as concentrated solutions were sterilized by filtration.

The shake flasks contained about 100 ml media initially out of 250 ml total volume. They were incubated at 32-34 ^{o}C. from 49 to 373 hours in a shaker set at 100 rpm. There was no control over pH, dissolved oxygen, or evaporation losses of ethanol. Samples were taken periodically and analyzed for cell mass by optical density and filtration, glucose by DNS, and ethanol by gas chromatography.

5.3.1.3 Results and Discussion

As seen in Figures 5.2 and 5.3, the stationary growth phase can be reached even without growth factors. However, the time to reach the stationary growth phase for medium without any growth factors or without biotin is 220 - 300 hours as compared to about 40 hours for the other media depicted in Figures 5.4 and 5.5. The stationary phase is evidenced by relatively constant optical density readings and total sugar utilization. Therefore, growth factors, either as synthetic vitamins or as yeast extract components, are stimulatory rather than essential to the growth of Saccharomyces cerevisiae, ATCC No. 4126.

Furthermore, the growth curves for biotin free and growth factor free media in Figure 5.2, and to less extent in Figure 5.3, closely

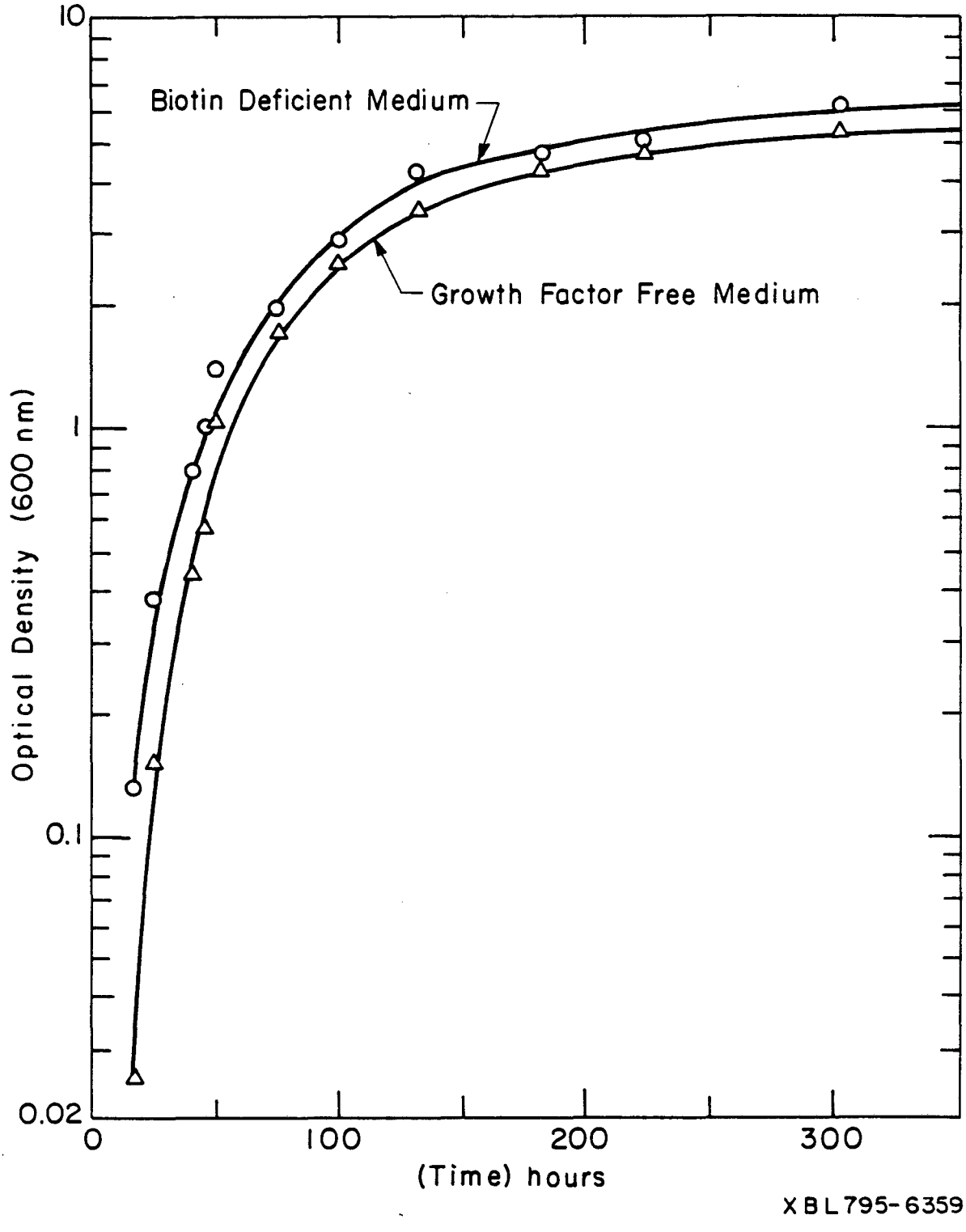

Figure 5.2 Effect of Biotin-free and Growth Factors-free Media (4-1 and 4-8) on Yeast Growth

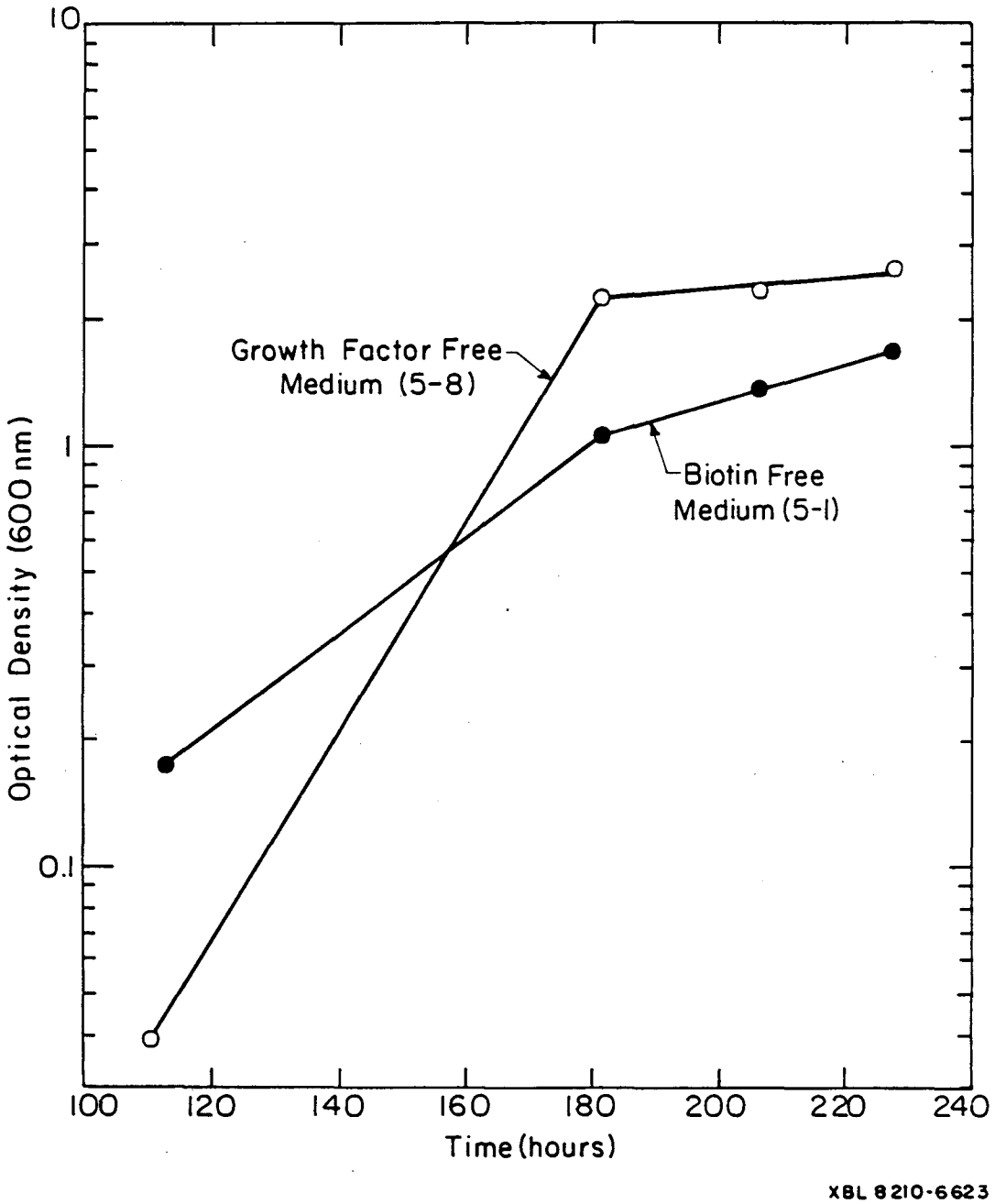

Figure 5.3 Effect of Biotin-free and Growth Factors-free Media (5-1 and 5-8) on Yeast Growth

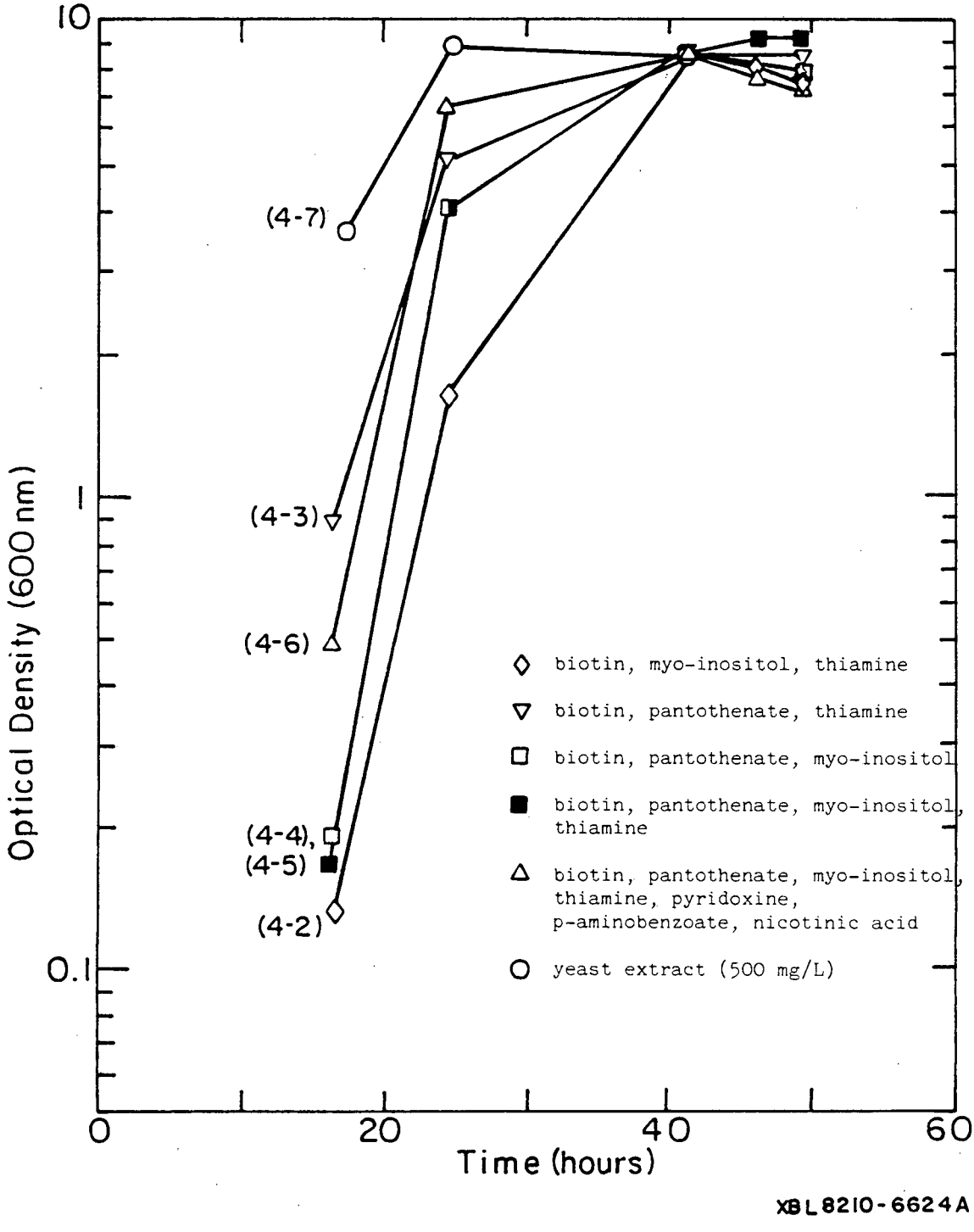

Figure 5.4 Effect of Various Growth Factor Combinations on Yeast Growth in Shake Flask Set 4

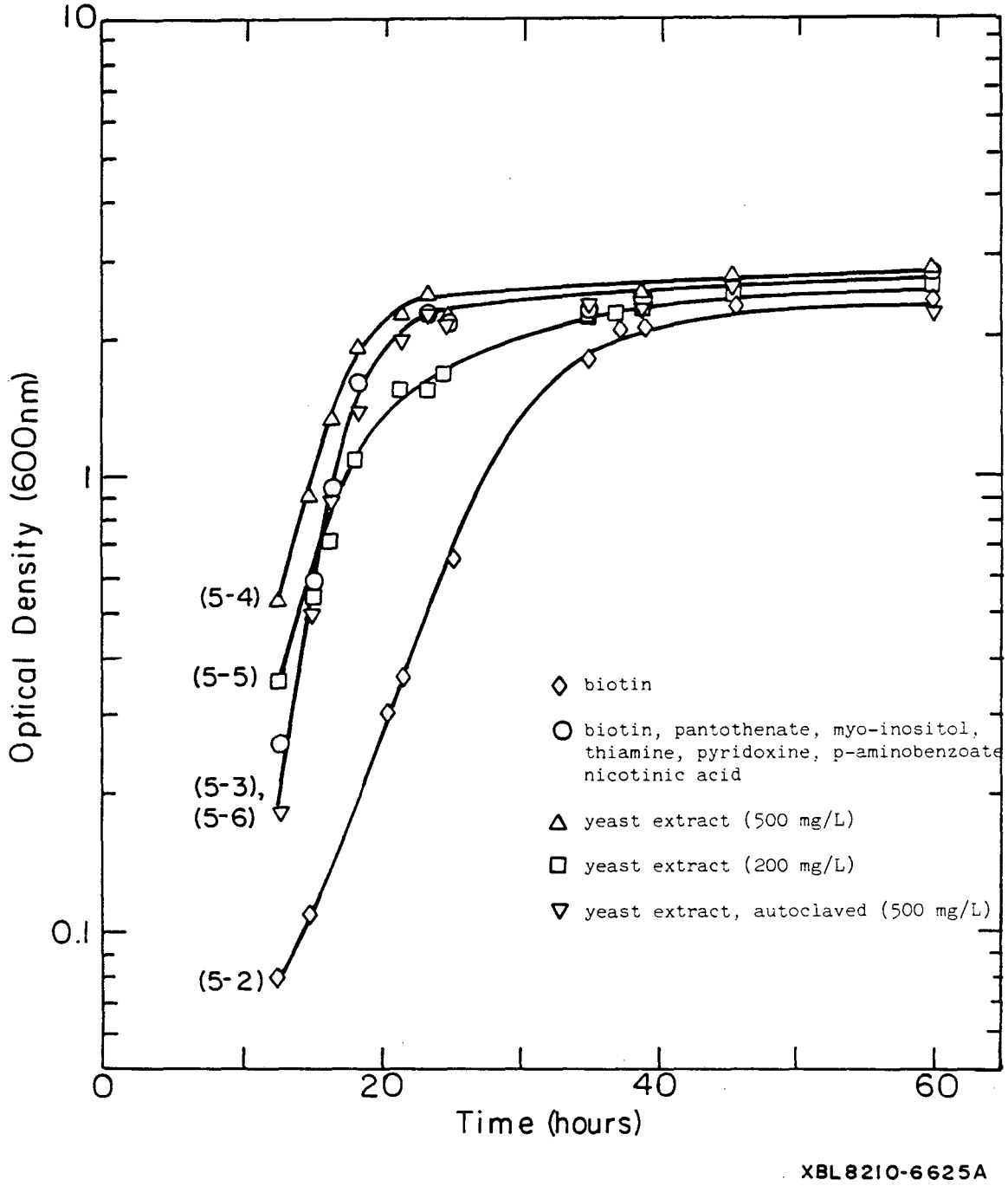

Figure 5.5 Effect of Various Growth Factor Combinations on Yeast Growth in Shake Flask Set 5

parallel each other. The ethanol production curves in Figure 5.6 for biotin free (Medium 4-1) and growth factor free (Medium 4-8) media are also parallel during the exponential phase. Thus, it appears that biotin is the rate limiting growth factor with respect to cell growth and ethanol production. As a corollary, the other growth factors are not rate limiting unless the biotin requirement is satisfied.

The maximum specific growth rates for the growth curves in Figures 5.4 and 5.5 are summarized in Table 5.6. Except for the biotin free and growth factor free media growth curves, the growth rates are not significantly different, implying that the growth rates of synthetic growth factor media are comparable to those of yeast extract media in these cases. The effect of autoclaving is not discernible from these shake flask cultures.

As seen in Figure 5.6, medium with biotin as the only growth factor (Medium 5-2) appears to produce ethanol significantly slower than the media with biotin and other growth factors or with yeast extract. Furthermore, the Medium 5-2 maximum specific growth rate and glucose consumption rate are lower than those for media with additional growth factors or with yeast extract as seen in Figures 5.5 and 5.7. With biotin present, another growth factor appears to be rate limiting with respect to ethanol production and possibly growth.

5.3.2 Batch Fermentor (Miniferm) Studies

Three sets of batch fermentations, designated as MF1, MF2, and MF3, were carried out in one liter Miniferm fermentors to provide more accurate data than were obtainable with the shake flasks, especially for

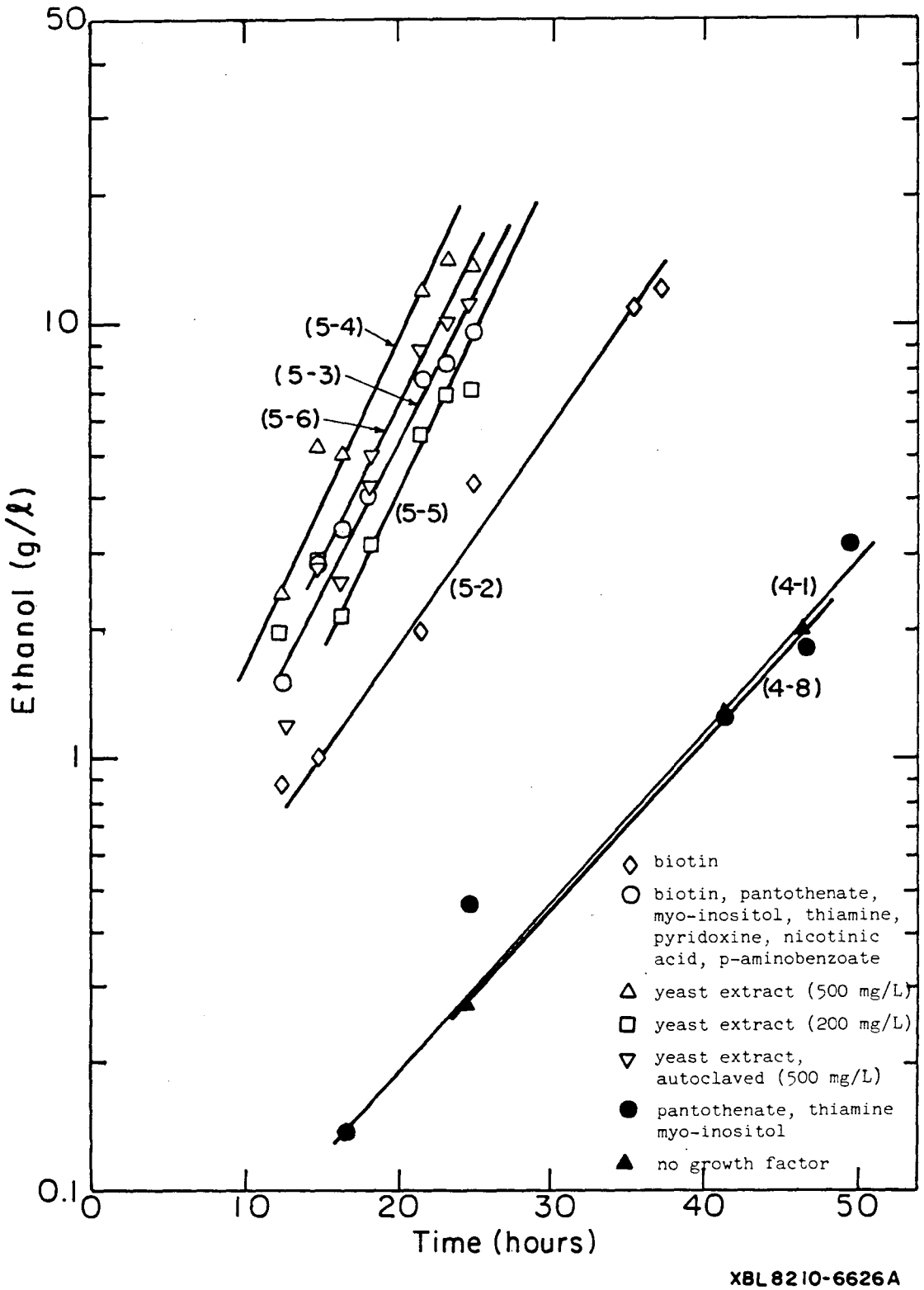

Figure 5.6 Effect of Various Growth Factor Combinations on Ethanol Production Rates in Shake Flask Sets 4 and 5

Table 5.6

Summary of Maximum Specific Growth Rates for Shake Flask Sets 4 and 5

Shake Flask Medium		μ_{max}
No.	Growth Factors *	(1/hr)
4-1	pant, inos, thia	0.071
4-2	biot, inos, thia	0.32
4-3	biot, pant, thia	0.22
4-4	biot, pant, inos	0.40
4-5	biot, pant, inos, thia	0.38
4-6	biot, pant, inos, thia, pyri, paba, nico	0.32
4-7	yeast extract (500 mg/L)	
4-8	no growth factors	0.063
5-1	pant, inos, thia, pyri, paba, nico	0.026
5-2	biot	0.18
5-3	biot, pant, inos, thia, pyri, paba, nico	0.32
5-4	yeast extract (500 mg/L)	0.22
5-5	yeast extract (200 mg/L)	0.19
5-6	autoclaved yeast extract (500 mg/L)	0.35
5-7	autoclaved version of Medium 5-3	
5-8	no growth factors	0.059

* Abbreviations:

biot = d-biotin
pant = Ca d-pantothenate
inos = myo-inositol
thia = thiamine HCl
pyri = pyridoxine HCl
paba = Na p-aminobenzoate
nico = nicotinic acid

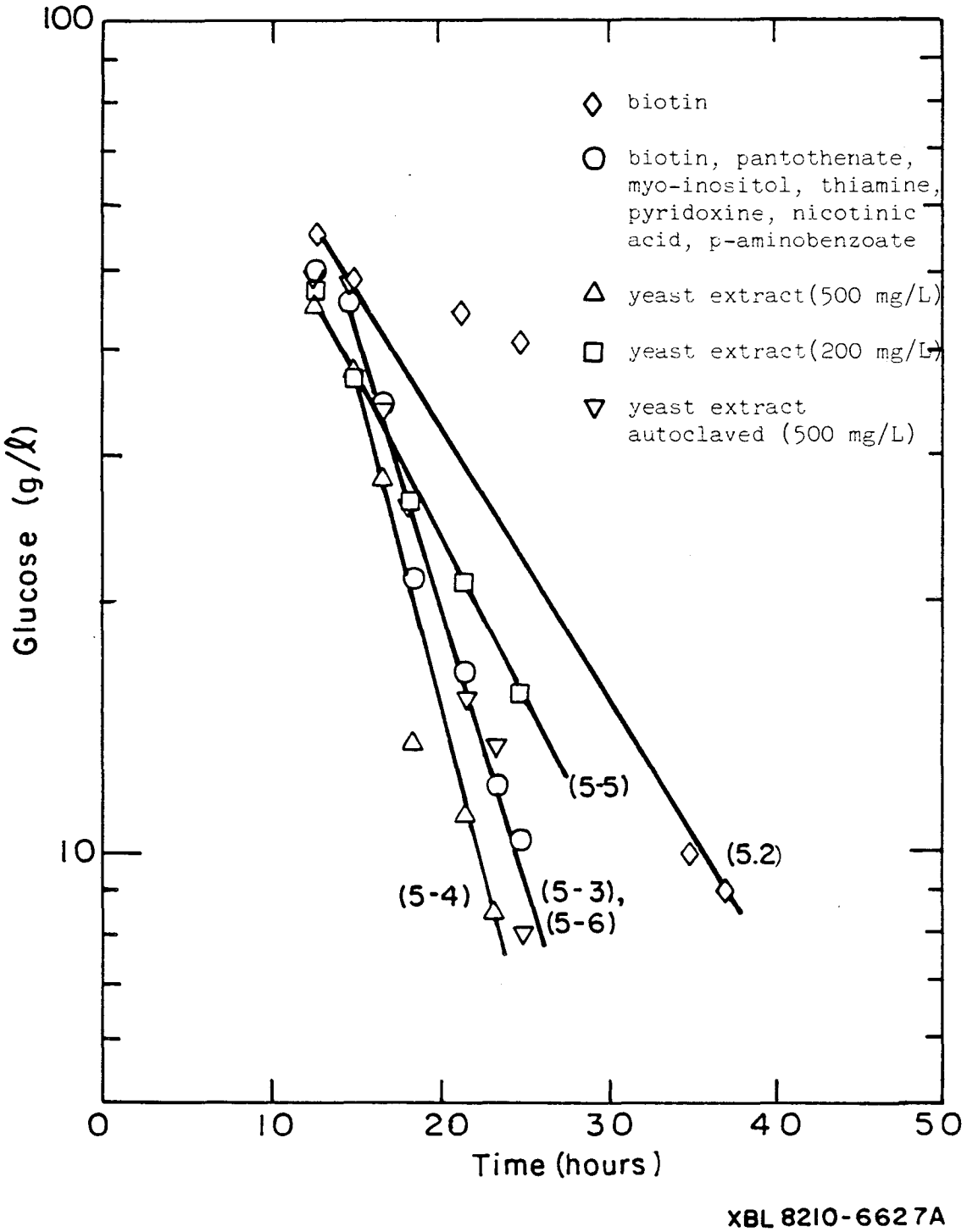

Figure 5.7 Effect of Various Growth Factor Combinations on Glucose Consumption Rates in Shake Flask Set 5

ethanol yield and rates.

5.3.2.1 Media Studied

The media compositions studied are given in Tables 5.7, 5.8, and 5.9. The MF1 Miniferms were run to determine if a synthetic growth factor mixture could replace yeast extract and to determine the effect of biotin as the only growth factor in the medium.

The MF2 Miniferms were run to determine the effect of increasing the yeast extract concentration from 0.5 to 4.25 g/L. In addition, the effect of doubling the glucose concentration to 100 g/L (and doubling all the other components to keep the same ratio of components) was studied with MF2-4.

The MF3 Miniferms attempted to determine the effects of varying synthetic growth factor concentrations and of adding certain amino acids. The base level of growth factors in these Miniferms were increased 4 times (except 3.2 times for inositol) those of the previous growth factors supplemented Miniferm (MF1-1) to make the growth factors used less likely to be rate limiting. The amino acids were added to determine if they were responsible for the slightly higher specific growth rate of yeast extract supplemented MF1-3 relative to synthetic growth factors supplemented MF1-1. The amino acids selected for addition were asparagine, glutamine, serine, and threonine. This group of amino acids was selected because of their high rate of absorption relative to other amino acids from wort by top fermenting yeast (1,2). Furthermore, yeast growth was observed to be faster in wort, which contained amino acids, than in medium with ammonium as the only nitrogen

Table 5.7

Media for Miniferm Fermentor Set 1 (MF1)

Base Medium Same as in Table 5.1

Growth Factors	Media (mg/L)		
	MF1-1	MF1-2	MF1-3
d-biotin	0.50	0.50	
Ca d-pantothenate	6.25		
myo-inositol	125.		
thiamine HCl	5.0		
pyridoxine HCl	6.25		
Na p-aminobenzoate	1.0		
nicotinic acid	5.0		
yeast extract			500.

Table 5.8

Media for Miniferm Fermentor Set 2 (MF2)

Component	Media (g/L)			
	MF2-1	MF2-2	MF2-3	MF2-4
Glucose (J.T. Baker reagent grade)	50.	50.	50.	100.
$(NH_4)_2SO_4$	5.19	5.19	5.19	10.4
KH_2PO_4	1.53	1.53	1.53	3.06
$MgSO_4 \cdot 7H_2O$	0.55	0.55	0.55	1.10
$CaCl_2 \cdot 2H_2O$	0.13	0.13	0.13	0.26
H_3BO_3	0.01	0.01	0.01	0.02
$ZnSO_4 \cdot 7H_2O$	0.01	0.01	0.01	0.02
$CuSO_4 \cdot 5H_2O$	0.004	0.004	0.004	0.008
$MnSO_4 \cdot H_2O$	0.003	0.003	0.003	0.006
$Al_2(SO_4)_3$	0.003	0.003	0.003	0.006
$FeSO_4 \cdot 7H_2O$	0.002	0.002	0.002	0.004
$CoSO_4 \cdot 7H_2O$	0.001	0.001	0.001	0.002
KI	0.001	0.001	0.001	0.002
yeast extract	0.5	2.0	4.25	1.0

Table 5.9

Media for Miniferm Fermentor Set 3 (MF3)

Base Medium Composition: double concentrations in Table 5.1 (same as MF2-4 without yeast extract)

Growth Factors	Media (mg/L)			
	MF3-1	MF3-2	MF3-3	MF3-4
d-biotin	2.0	5.0	2.0	2.0
Ca d-pantothenate	25.	62.	25.	25.
myo-inositol	400.	1000.	400.	400.
thiamine HCl	20.	50.	20.	20.
pyridoxine HCl	25.	62.5	25.	25.
Na p-aminobenzoate	4.0	10.	4.0	4.0
nicotinic acid	20.	50.	20.	20.
Amino Acids				
asparagine			200.	1000.
glutamine			200.	1000.
serine			50.	250.
threonine			50.	250.

source (3). Thus, the selected amino acids appear to be highly preferred nitrogen sources.

5.3.2.2 Procedure

A schematic of the Miniferm setup for batch fermentations is shown in Figure 5.8. The Miniferm fermentors have total volumes of about 1000 ml, but their working volumes were approximately 750 ml, 500 ml, and 755 ml in MF1, MF2, and MF3, respectively. The fermentor temperature was controlled at 35 ^{o}C. by a constant temperature water bath. Mixing was provided by a stirring bar driven by a magnetic stirrer.

After sterilization by 0.22 micron filters, all media were sparged with air for about two hours to ensure air saturation before inoculation. No further oxygen was supplied after inoculation. In fact, nitrogen was used to sweep the fermentor headspace during sampling to reduce the probability of contamination. Cysewski (4) had found the optimal method of oxygen supply to 100 g/L glucose medium for batch yeast growth to be air saturation of the pre-inoculated broth and inoculation with an aerobically grown inoculum. Therefore, even without dissolved oxygen control in the Miniferm fermentations, the growth and ethanol production rates should not be adversely affected.

The pH was also not controlled in the Miniferm runs. Using the same yeast, Cysewski reported a broad optimal pH range from 4.0 to 5.5 (4). The initial pH of the Miniferm media was between 4.60 to 5.55. Furthermore, the pH appeared to have remained in the optimal range during the early exponential phases, when growth and ethanol production rates were determined. However, the pH decreased below the optimal range

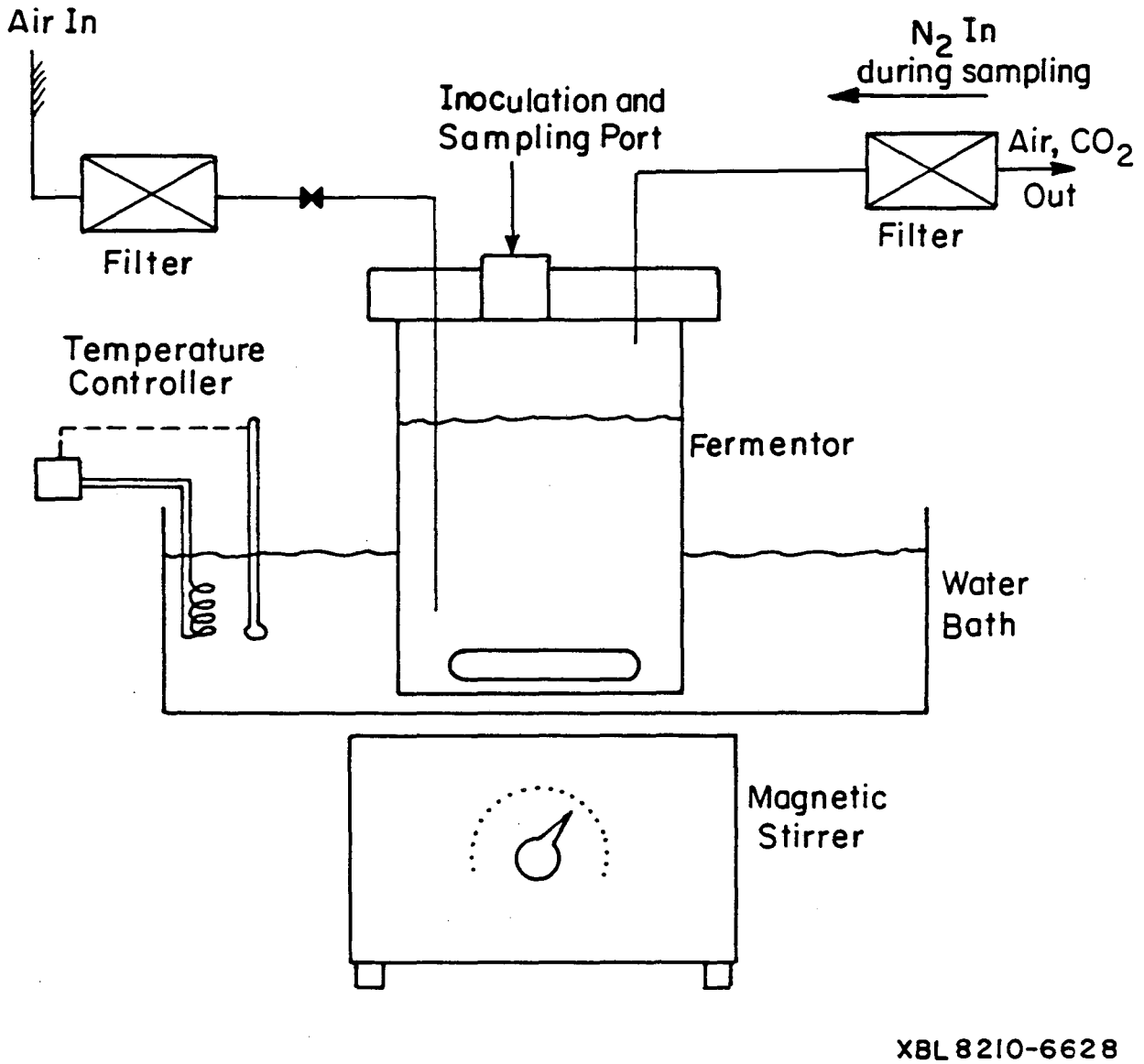

Figure 5.8 Miniferm Fermentor for Batch Cultures

toward the end of the exponential phase.

The inocula for all Miniferms were derived from shake flask cultures grown in the same medium as that used in the Miniferm. For minimal growth factors media, such as MF1-2 and MF1-3, the inocula were twice centrifuged and washed with fermentor medium before being used. This procedure should have minimized carry over of extraneous growth factors. Washing of the inocula for Miniferm Sets 2 and 3 was not considered necessary. The inoculum for the inocula shake flasks for MF2 and MF3 were grown in Media MF2-1 and MF3-1, respectively. Media MF2-1 and MF3-1 had much lower concentrations of all growth factors relative to the other media in their respective sets.

Samples were taken with sterile pipettes from all Miniferms approximately every hour during the exponential growth phase. Formaldehyde was added to the samples from MF2 and MF3 to stop further reactions. However, this practice was discontinued because formaldehyde was found to interfere with the DNS assay of reducing sugars. The sampling time was considered short enough not to permit significant further reactions without a quenching agent. Reducing sugars were measured by DNS for just MF1. Ethanol was measured by gas chromatography, and dry cell mass was measured by calibrated optical density.

5.3.2.3 Results and Discussion

Cell growth, ethanol production, and glucose consumption as functions of time are presented in Figures 5.9 through 5.15 for the batch Miniferm fermentations. The maximum specific growth rates (μ_{max}), maximum specific ethanol productivity (q_{pmax}), and other data from these

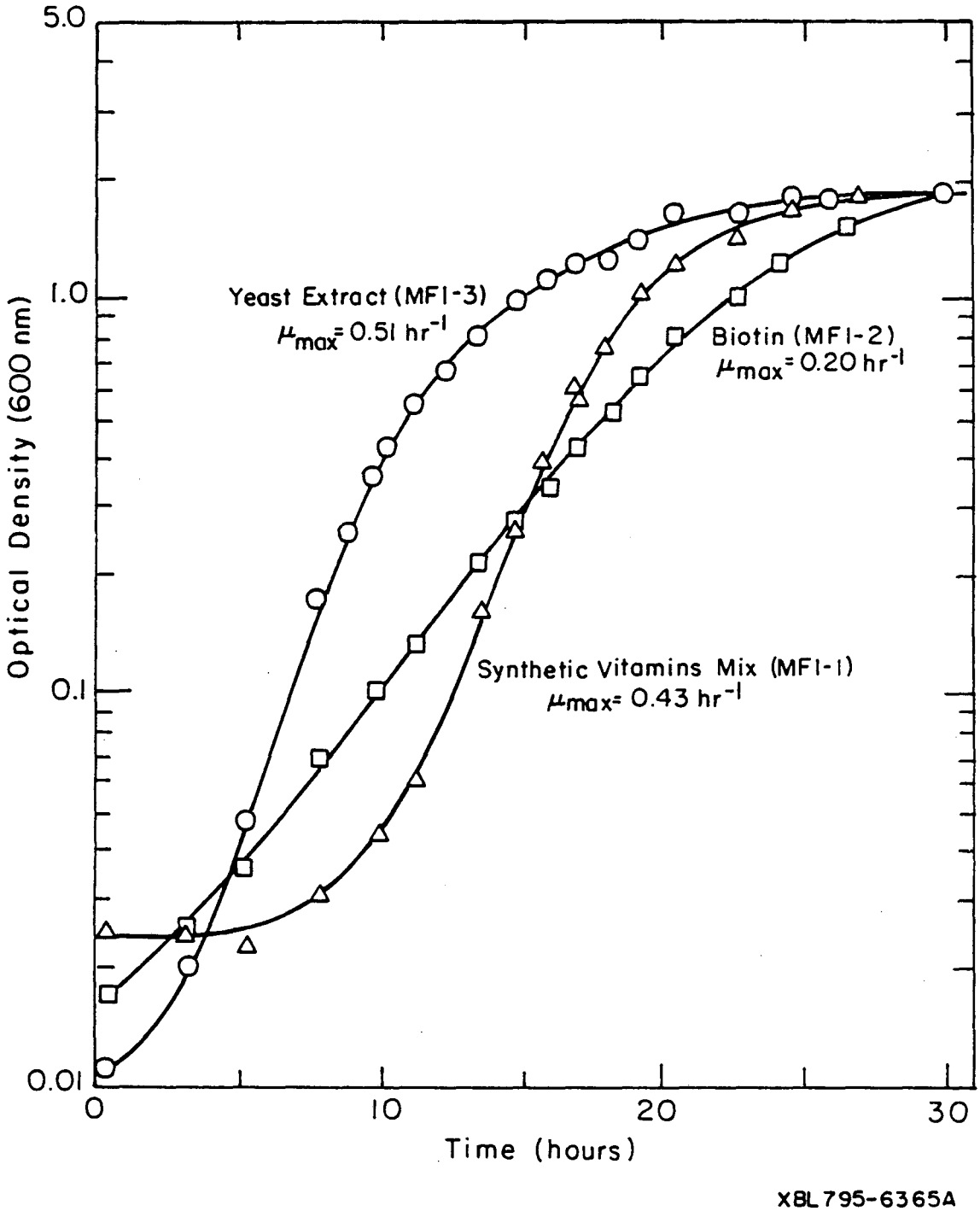

Figure 5.9 Comparison of Maximum Specific Growth Rates for Complex (Yeast Extract) versus Synthetic (Biotin Only and Complete Synthetic Vitamin Mix) Growth Factors in Miniferm Set 1

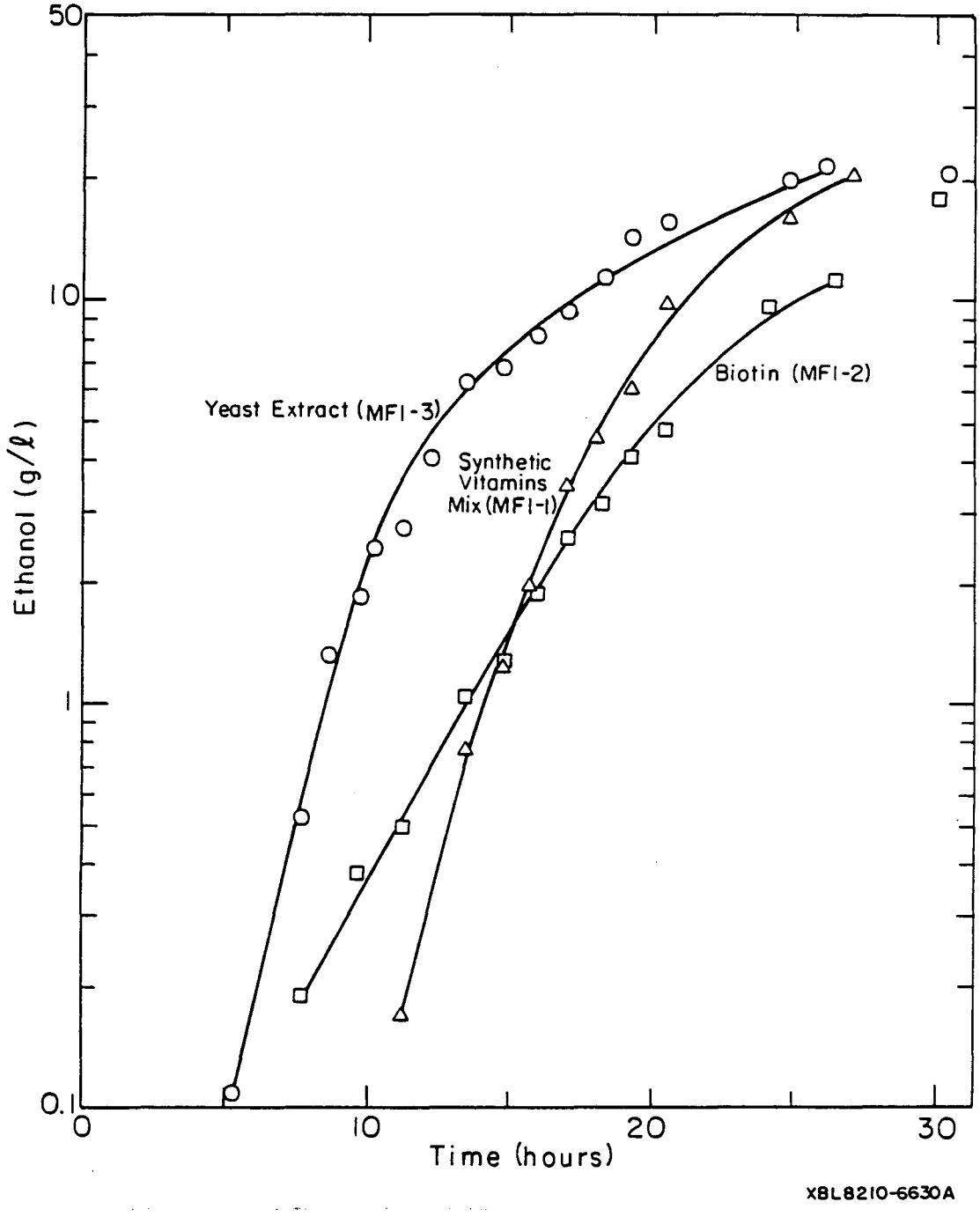

Figure 5.10 Comparison of Ethanol Production Rates for Complex (Yeast Extract) versus Synthetic (Biotin Only and Complete Synthetic Mix) Growth Factors in Miniferm Set 1

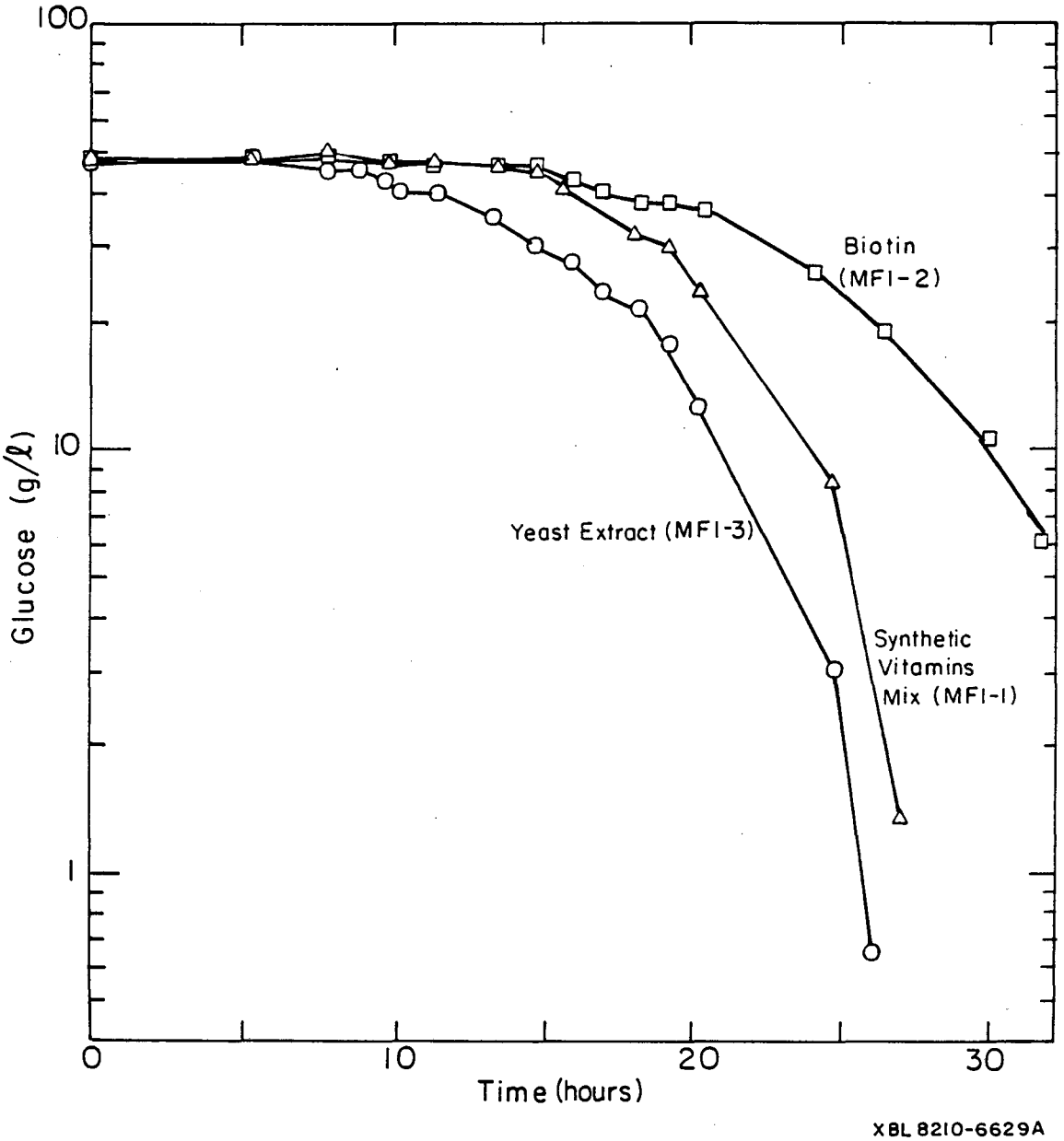

Figure 5.11 Comparison of Glucose Consumption Rates for Complex (Yeast Extract) versus Synthetic (Biotin Only and Complete Synthetic Mix) Growth Factors in Miniferm Set 1

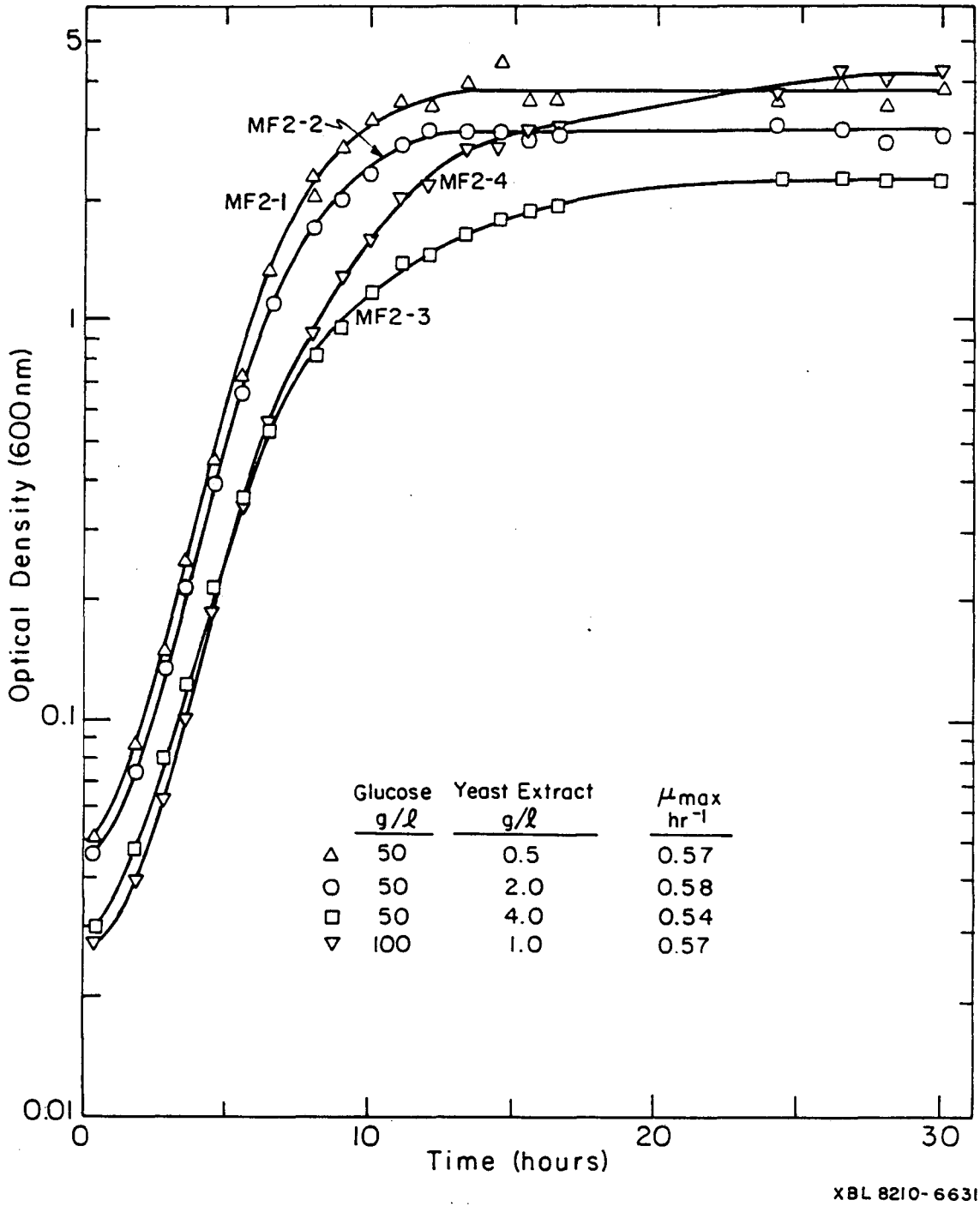

Figure 5.12 Effect of Yeast Extract Concentration on Yeast Growth Rates in Miniferm Set MF2

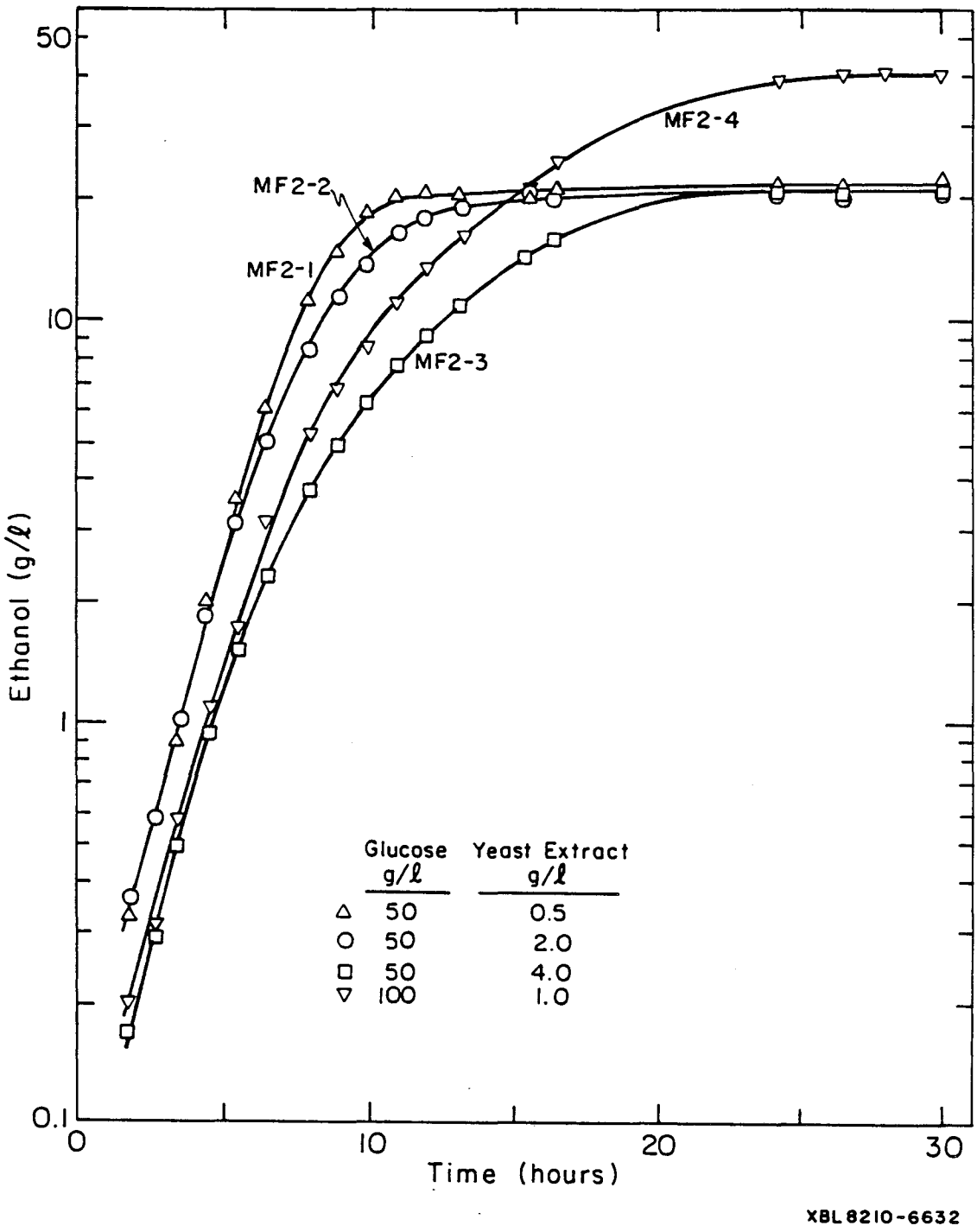

Figure 5.13 Effect of Yeast Extract Concentration on Ethanol Production Rates in Miniferm Set MF2

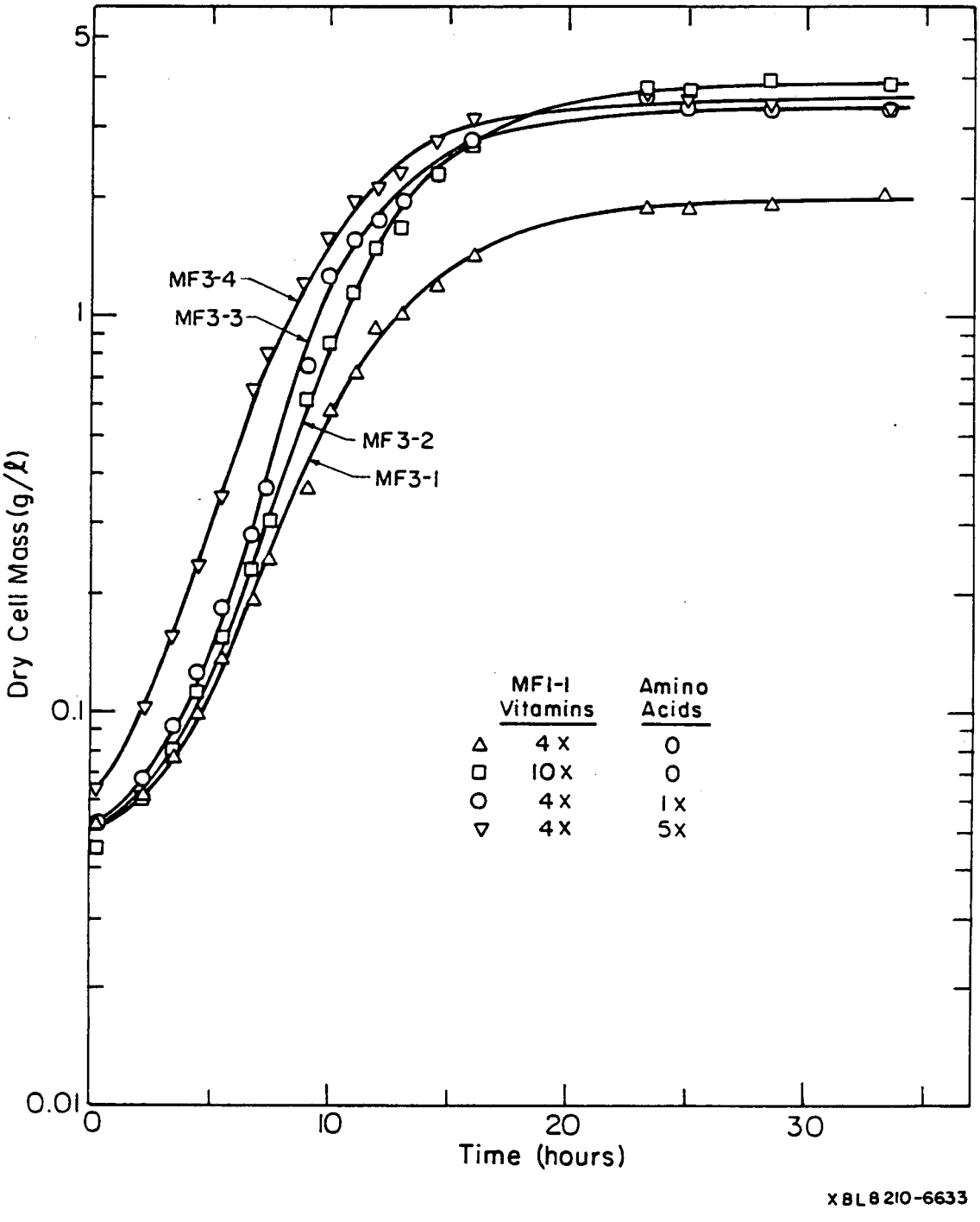

Figure 5.14 Effect of Vitamins and Amino Acids on Yeast Growth in Miniferm Set MF3

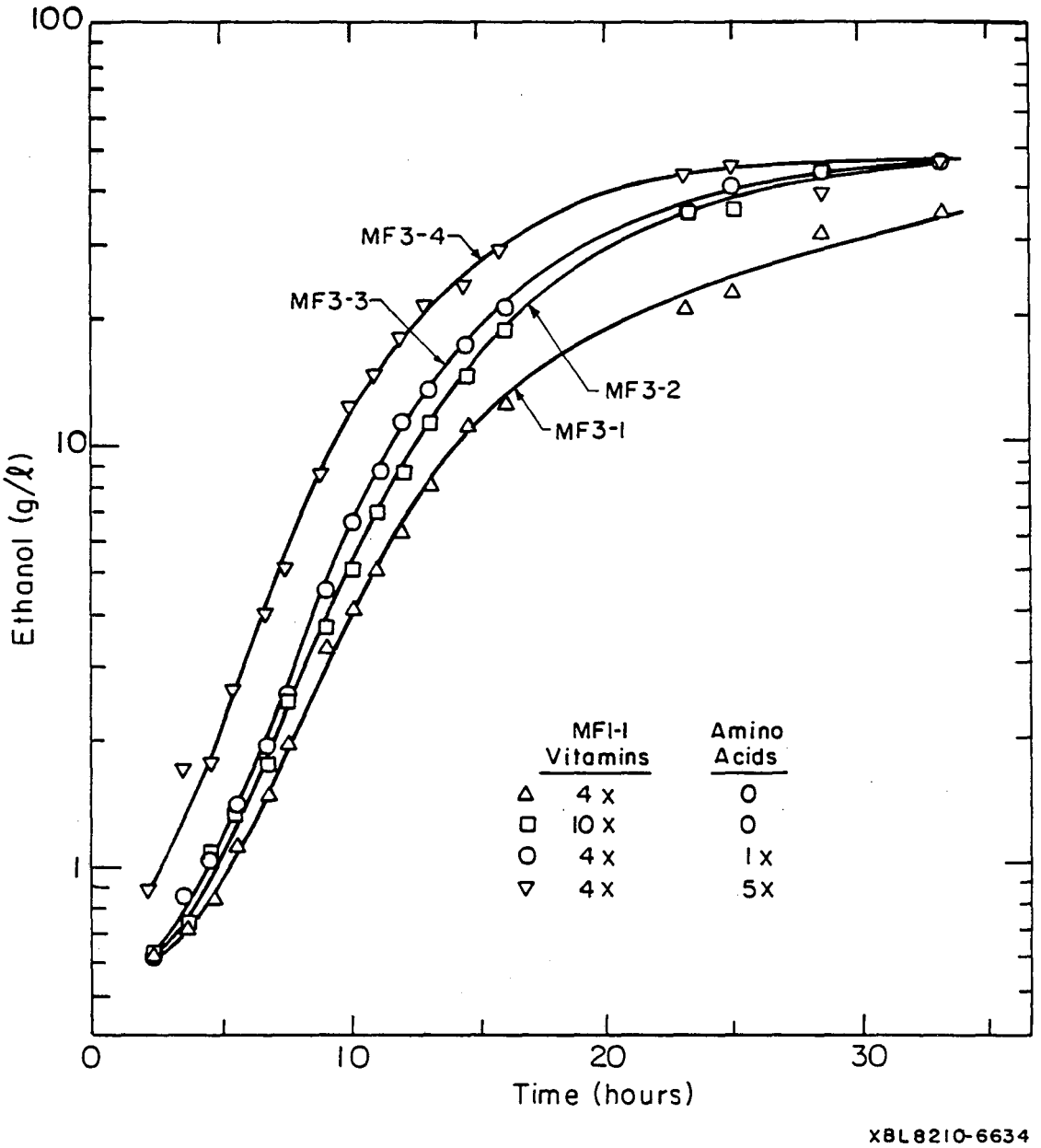

Figure 5.15 Effect of Vitamins and Amino Acids on Ethanol Production Rates in Miniferm Set MF3

figures are summarized in Table 5.10. Because many of the q_{pmax} values were determined at very low concentrations of cell mass and ethanol, their absolute values are subject to systematic analytical errors, but their relative values should be meaningful.

5.3.2.3.1 Comparison of Synthetic Growth Factors and Yeast Extract (Miniferm 1)

As seen in Figures 5.9, 5.10 and Table 5.10, 0.5 g/L yeast extract in the medium gives a slightly higher maximum specific growth rate, 0.51/hr, and also a higher q_{pmax}, 2.7 g-ethanol/g-cell-hr, than those of the synthetic vitamin mix, namely, 0.43/hr and 2.1 g-ethanol/g-cell- hr, respectively. Hence, in yeast extract there are important unaccounted for factors which are absent from the synthetic medium and are somewhat limiting to growth and ethanol production. The glucose consumption curves for these two media in Figure 5.11 appear approximately parallel after the initial decline phase, consistent with the ethanol results. An important difference between these two media in batch growth is the greater lag time for the synthetic vitamin mix. However, this lag phase should not be a factor in continuous culture.

The biotin only growth factor medium shows considerably slower maximum specific growth and ethanol production rates than the more complete growth factors medium. Thus, growth factors in addition to biotin are needed to stimulate rates, confirming the shake flask results.

5.3.2.3.2 Effect of Yeast Extract and Glucose (Miniferm 2)

Figures 5.12 and 5.13 indicate that the maximum specific growth

Table 5.10

Summary of Cell Mass and Ethanol Production
Rate and Yield Data from Batch Miniferm Cultures

Medium No.	Initial Glucose g/L	Yeast Extract g/L	Growth Factors g/L	Amino Acids g/L	μ_{max} 1/hr	Cell Mass g/L	Cell Yield $Y_{x/s}$	Maximum q_p 1/hr	Ethanol g/L	Ethanol Yield $Y_{p/s}$
MF1-1	50.		0.15		0.43	2.3	0.045	2.1	21.	0.41
MF1-2	50.		0.0005 Biotin only		0.20	2.3	0.045	1.6	21.	0.42
MF1-3	50.	0.5			0.51	2.3	0.046	2.7	21.	0.41
MF2-1	50.	0.5			0.54	2.7	0.054	2.3	22.	0.44
MF2-2	50.	2.0			0.58	3.6	0.072	2.4	22.	0.44
MF2-3	50.	4.2			0.57	4.1	0.081	2.5	22.	0.45
MF2-4	100.	1.0			0.57	4.7	0.047	3.0	43.	0.43
MF3-1	100.		0.50		0.33	2.2	0.022	1.9	45.	0.45
MF3-2	100.		1.2		0.40	4.0	0.040	2.1	44.	0.44
MF3-3	100.		0.50	0.5	0.47	3.4	0.034	2.2	44.	0.44
MF3-4	100.		0.50	2.5	0.45	3.6	0.036	2.0	46.	0.46

rates, 0.54 - 0.58/hr, and the maximum specific ethanol production rates, 2.3 - 2.5/hr, are remarkably uniform and independent of the yeast extract concentration from 0.5 to 4.25 g/L for 50 g/L glucose concentration. Although the initial concentrations of all the components of the 100 g/L glucose medium are double the concentrations of the 50 g/L glucose medium, their maximum specific growth rates are very close. The value of q_{pmax}, however, is greatest for the combination of 100 g/L glucose concentration and 1.0 g/L yeast extract. Thus, glucose inhibition is insignificant at 100 g/L, and ethanol inhibition is insignificant through the early exponential growth period with this initial glucose level.

In comparably controlled batch cultures with 8.5 g/L yeast extract for 100 g/L glucose, Cysewski's (4) maximum specific growth rate was 0.46/hr and q_{pmax} was 1.8 g-ethanol/g-cell-hr. With respect to rates, batch results indicate the feasibility of reducing Cysewski's yeast extract to glucose ratio from 0.085 to 0.01 with additional minerals in the medium. The low level requirement of yeast extract indicates that it is being used either as a catalytic growth factor and/or being assimilated as a micronutrient. As discussed in Chapter 1, yeast extract would not be used as a minerals source for industrial fermentations. Thus, explicit sources of minerals, such as phosphorous, potassium and ammonium salts need to be added to the medium if the yeast extract concentration is to be reduced.

5.3.2.3.3 Effect of Synthetic Growth Factors and Amino Acids (Miniferm 3)

Figures 5.14 and 5.15 show the effect of increased concentrations of synthetic growth factors and amino acids without yeast extract on cell mass and ethanol production rates. Increasing the growth factor concentration 2.5 fold and adding 0.5 g/L of amino acids in separate fermentations appeared to have increased the maximum specific growth rates, but appeared not to have affected q_{pmax}. The amino acids selected were expected to increase the growth rate because of their high rate of assimilation as carbon and nitrogen sources. Apparently, 0.5 g/L provided a saturation level of these amino acids, since an increase to 2.5 g/L produced no further significant effects.

5.4 Extended Study of the Growth Factor Effects on Cell Mass and Ethanol Yields

5.4.1 Shake Flask Studies

The shake flask study discussed in section 5.3.1 with respect to rate effects (Set 5) also produced additional data with respect to cell mass yields. The media composition and the experimental procedure for this shake flask study are given in sections 5.3.1.1 and 5.3.1.2, respectively.

The cell mass yield data from this shake flask study were obtained at the end of batch growth in the stationary phase and are presented in Table 5.11. The only major discrepancy between cell mass yield data from this study and the previous Shake Flask Sets 1 - 3 was with medium not containing growth factors (Medium 5-8). The much higher cell yield of Medium 5-8 may have been due to a small carry over of growth factors with its inoculum since it did not go through the extensive subculturing process of Shake Flask Sets 1 - 3.

Table 5.11

Cell Yield Data from Shake Set 5

Shake Flask Medium No.	Growth Factors*	$Y_{x/s}$ (g-cells/g-glucose consumed)
5-1	pant, inos, thia, pyri, paba, nico	0.030
5-2	biot	0.053
5-3	biot, pant, inos, thia, pyri, paba, nico	0.055
5-4	yeast extract (500 mg/L)	0.054
5-5	yeast extract (200 mg/L)	0.055
5-6	autoclaved yeast extract (500 mg/L)	0.053
5-7	autoclaved version of Medium 5-3	
5-8	no growth factors	0.054

*Growth factor abbreviations same as in Table 5.6

The other media common to Shake Flask Sets 1 - 3 and 5 are medium lacking all growth factors (5-8), medium containing seven synthetic growth factors (5-3), and medium containing 500 mg/L yeast extract (5-4). For these media the cell mass yields showed good agreement with the previous shake flask studies. As seen previously, biotin is the most critical growth factor with respect to cell yield as evidenced by the low yield of medium lacking biotin (5-1). With biotin or yeast extract present the cell yield is remarkably uniform between 0.053 to 0.055. Autoclaving the yeast extract had negligible effect on cell yield.

5.4.2 Batch Fermentor (Miniferm) Studies

The cell mass and ethanol yield data were derived from the same batch fermentations used for rate studies in section 5.3.2 and are summarized in Table 5.10. The much higher ethanol yields, $Y_{p/s}$ = 0.41 - 0.46, obtained in the Miniferms, indicate the previously obtained ethanol yields for the shake flask studies in section 5.2 are probably too low. However, the relative results within a given shake flask set still have validity.

Cell mass and ethanol yields are practically the same in medium containing 500 mg/L yeast extract, 0.5 mg/L biotin, and 149 mg/L mixed synthetic growth factors. Thus, biotin appears to be the only growth factor requirement which must be satisfied to obtain the yields reported in MF1 studies. The ethanol yields for all the batch Miniferm media are not significantly different.

Increasing the yeast extract in the medium increased the cell mass yield but not the ethanol yield. The increased cell mass reflected the

assimilation of amino acids and precursors from the yeast extract into the cell without sgnificant production of ethanol or its associated energy. The sugar content of yeast extract was too low to significantly affect the ethanol yield. Cysewski's (4) comparable batch fermentations confirmed that cell yield increased, but ethanol yield was constant with increased yeast extract.

The 100 g/L glucose media (MF2-4 and MF3) indicated that the ethanol yield was not affected by the increased ethanol relative to that from 50 g/L glucose. However, the cell yield appeared to have been reduced by this increased ethanol. Increasing the growth factors and amino acids concentrations did not affect the ethanol yield and probably also not the cell mass yield. The lack of effect on cell mass yield assumed that the cell yield of 0.022 for MF3-1 was incorrectly low based on previous Miniferm and shake flask results.

5.5 Conclusions

Saccharomyces cerevisiae ATCC 4126 is able to grow and produce ethanol in a completely synthetic media without growth factors. However, the cell mass yield is lower and the growth and ethanol production rates are much slower. The growth factors are, therefore, stimulatory to growth and ethanol production rates and also critical to cell yield.

The most important growth factor is biotin. The growth rate in biotin deficient medium containing six other growth factors, namely, pantothenate, thiamine, pyridoxine, inositol, nicotinic acid and p-aminobenzoic acid, is roughly the same as in medium without any growth factors. Thus, biotin appears to be the first growth rate limiting

growth factor. Media deficient in the other six growth factors in various combinations, but with biotin present, produce considerably faster growth rates than biotin deficient media. However, other growth factors are required for maximum growth and ethanol production rates. Biotin also appears to be the only critical growth factor with respect to cell mass and ethanol yields. It is the only growth factor whose absence produced consistently low cell mass and ethanol yields. Furthermore, biotin as the only growth factor is sufficient to produce cell and ethanol yields comparable to those from the set of seven growth factors or from yeast extract.

Synthetic growth factors supplemented media with or without amino acids can attain maximum specific growth and ethanol production rates comparable to those obtained by Cysewski (4) with relatively high concentrations of yeast extract. Even higher maximum specific growth rates can be achieved with this synthetic growth factors medium supplemented with relatively low concentrations of yeast extract. Increasing the yeast extract above this minimum level does not appear to affect the maximum specific growth rate or the ethanol yield, but increases the cell yield. The addition of the four amino acids, asparagine, glutamine, serine and threonine, does not appear to improve the yields or rates of production of cells or ethanol.

Quantitative determination of the optimum composition of the growth factors and other medium components require well controlled continuous cultures in which each nutrient can be varied individually with the medium and environment otherwise constant. The growth factors composition in Table 5.12 represent an upper bound on the growth factors

Table 5.12

Upper Bounds on Growth Factor Requirements for Fermentation of 100 g/L Glucose Based on Batch Cultures

Growth Factor	Concentration (mg/L)
d-biotin	1.0
Ca d-pantothenate	12.5
myo-inositol	250.
thiamine HCl	10.0
pyridoxine HCl	12.5
Na p-aminobenzoate	2.0
nicotinic acid	10.0

requirement based on batch cultures and give a starting point for the continuous studies presented in the next chapters.

5.6 References for Chapter 5

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Chapter 6

6. Media Requirements for Low Glucose Feed Continuous Cultures

6.1 Introduction

The media components required for ethanol fermentation are identified in Chapter 5. Their approximate upper concentration requirements determined from batch fermentations are given in Table 5.12. The objective of this chapter is to establish minimal media requirements for glucose feed concentrations of 5 to 25 g/L in continuous cultures. This range is sufficiently low to eliminate significant inhibitory effects from ethanol, by-products, and excess substrates. In the next chapter, nutrient requirements are established for a glucose feed of 100 g/L, Cysewski's (1) optimum concentration for chemostat cultures. Continuous fermentations are desirable for nutritional studies because they allow all conditions except for the nutrient being studied to be held approximately constant and because they eliminate inoculum history effects.

6.2 Procedure

To determine minimal media requirements, a novel procedure, extending developments by Mateles and Battat (2), was employed. A schematic of the continuous fermentation setup is shown in Figure 6.1. Each medium component to be optimized is made limiting in terms of cell and ethanol production. The initial feed medium is made low in or devoid of the components to be studied as limiting nutrients in continuous culture. The limiting nutrients for this and subsequent media are determined by injecting concentrated pulses of each of the possible limiting

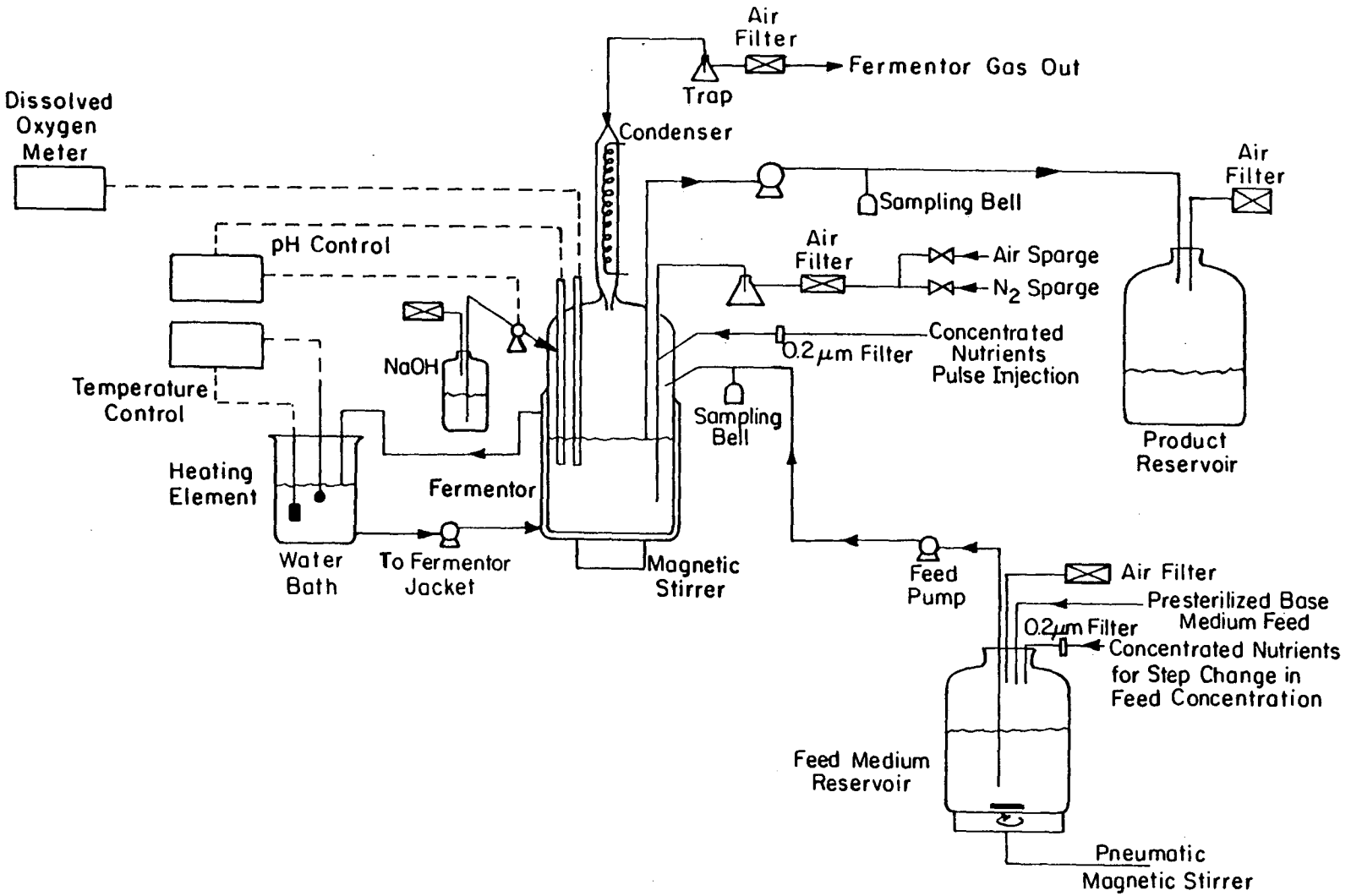

Figure 6.1 Continuous Fermentation Apparatus

nutrients in turn into the fermentor until a transient increase in cell mass or ethanol is observed. Increases in cell mass are normally accompanied by increases in ethanol in growth associated production. Thus, only cell mass by means of optical density are normally measured for transient responses.

Figure 6.2 shows the approximate responses of the fermentor glucose and cell mass concentrations to injections of limiting and non limiting nutrients. The fermentor glucose reponse to injection of glucose as a limiting nutrient is the superposition of the glucose pulse from the injection and the decrease in glucose from cell consumption.

Once a nutrient has been identified as limiting by this procedure, the minimal requirement of this nutrient with respect to cell mass and ethanol production can be determined. These are obtained from the ratios of the feed concentration of this limiting nutrient to the steady state concentrations of the fermentor cell mass and ethanol before injection.

After a limiting nutrient is found, its concentration in the feed reservoir is increased such that it is no longer limiting, as shown in Figure 6.3. The new feed concentration can be set by the approximate equation:

$$S_{R1} \approx (S_{R0} - S_0) \times (X_{desired}/X_0)$$

where

S_{R1} = new non-limiting nutrient feed reservoir concentration

S_{R0} = original reservoir concentration of limiting nutrient

S_0 = original fermentor concentration of limiting nutrient

X_0 = original fermentor cell mass concentration

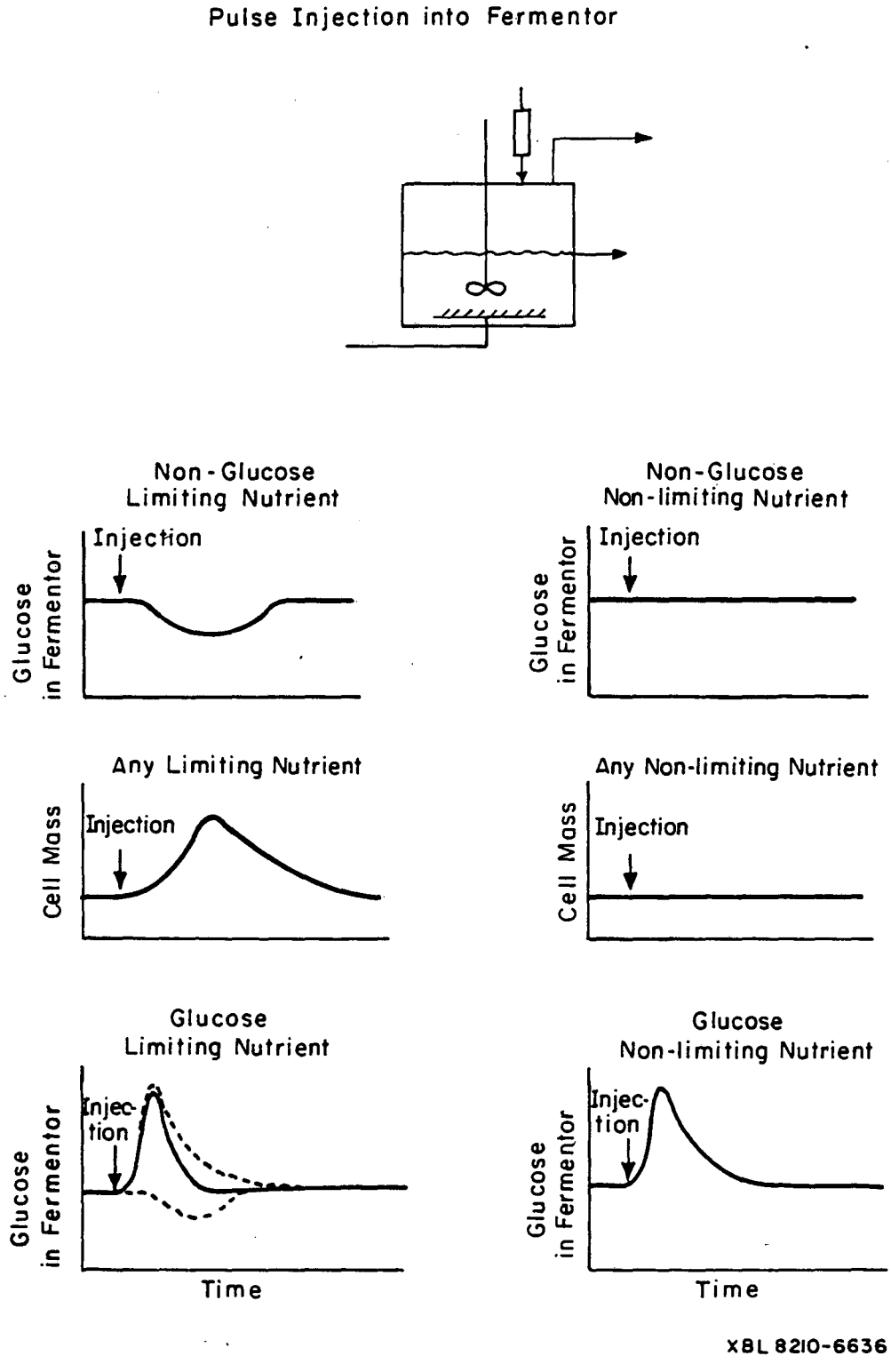

Figure 6.2 Transient Responses to Pulse Injections of Nutrients into Continuous Cultures

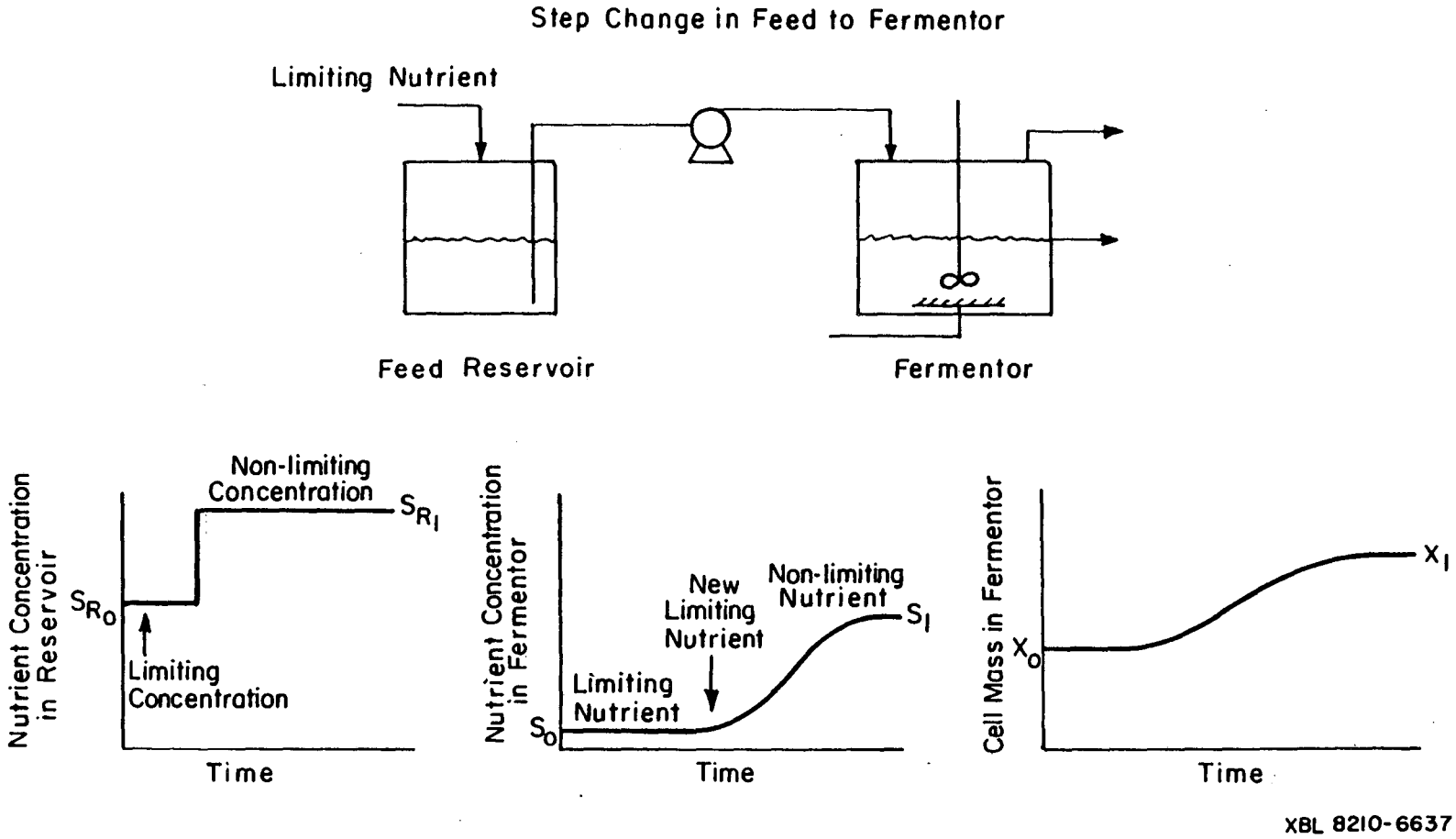

Figure 6.3 Steady State Response to Step Change in Feed Concentration of Nutrient in Continuous Culture

X_1 = new fermentor cell mass concentration following increase in feed concentration of previously limiting nutrient

$X_{desired}$ = desired fermentor cell mass concentration after all non glucose limiting nutrients are made non-limiting

The previously limiting components can then be eliminated from further testing as the other components in turn become limiting.

Except as noted, all continuous fermentations were controlled at 35 ^{o}C and pH 4.0 - 4.5. Dissolved oxygen concentration was monitored and controlled manually through the agitation rate and the inlet gas rate and composition. A condenser was used to minimize vapor losses from the fermentor. The refrigerant used in the condenser was normally kept at about 4 ^{o}C.

6.3 Initial Optimization - Search for Active Components of Yeast Extract

The minimal base medium for the initial continuous cultures with low glucose concentration feed is given in Table 6.1. To limit the search for limiting nutrients initially to major components, 0.1 g/L of yeast extract was included in the minimal base medium. A search was made later for active trace components of yeast extract not examined for in this study.

The New Brunswick Scientific Company fermentor used for this initial optimization study was 5 liters in total volume and 2.4 liters in working volume. It was equipped as described in Procedure section 6.2 except cool industrial water was used for the condenser on the fermentor. The dilution rate was maintained at 0.27/hr. Transient responses

Table 6.1

Low Glucose Continuous Culture

Minimal Base Medium for 10 g/L Glucose

Component	Concentration (g/L)
$(NH_4)_2SO_4$	0.472
KCl	0.0763
H_3PO_4	0.0821
$CaCl_2 \cdot 2H_2O$	0.0118
$MgSO_4 \cdot 7H_2O$	0.0495
Trace Elements Solution	0.5 ml/L

Trace Elements Solution

Component	Concentration (g/L)
H_3BO_3	2.0
$CoSO_4 \cdot 7H_2O$	0.2
$CuSO_4 \cdot 5H_2O$	0.8
$ZnSO_4 \cdot 7H_2O$	2.0
$MnSO_4 \cdot H_2O$	0.6
KI	0.2
$FeSO_4 \cdot 7H_2O$	0.4
$Al_2(SO_4)_3$	0.6

were expected after approximately three to six hours. Steady state responses were expected after twelve hours. The approximate cell mass was followed by the cell suspension optical density.

The conditions and results are summarized in Table 6.2. All media were formulated from the minimal base medium supplemented with the specified glucose, yeast extract and other nutrients. Media 1 and 2 showed that the initial limiting substrate for the minimal base medium is not glucose, trace elements, $(NH_4)_2SO_4$ or KCl. Medium 3 indicated that the limiting substrate was contained in yeast extract since a 3.5 fold increase in yeast extract increased the cell mass almost proportionally to 3.74 times. Additional yeast extract in Medium 4 did not significantly increase cell mass probably because the glucose had become depleted. Additional oxygen to Medium 4 increased the cell mass slightly at the expense of ethanol probably as a result of a slight shift from fermentation to respiration. The glucose concentration was too low to permit catabolite repression of respiration. The positive response to the pulsed glucose confirmed that glucose was limiting with the increased yeast extract in Medium 4.

After returning the feed yeast extract concentration to 0.1 g/L, the search focused on components of yeast extract as the limiting nutrient. Media 5,6, and 7 focused on the mineral requirements; H_3PO_4, $CaCl_2$, $MgSO_4$, NaCl, and all minimal base nutrients were increased without positive response. The search then turned to growth factors with Media 7,8, and 9. Myo-inositol, para-aminobenzoic acid, pyridoxine, nicotinic acid, thiamine, and pantothenic acid all produced no response. Increasing the biotin in the feed to 4.0 micrograms /liter

Table 6.2

Low Glucose Continuous Culture - Initial Optimization

Medium[1]	Glucose (g/L)	Yeast Extract (g/L)	Pulses & Shifts in Nutrients[3]	Cell O.D.	Ethanol (g/L)	Residual Glucose (g/L)	Response[2]
1	5.0	0.1		0.331	1.18	1.77	
2	10.0	0.1	2 X Trace Elements	0.326	1.19	6.47	None
			Pulse $(NH_4)_2SO_4$	0.325	1.34	6.49	None
			Pulse KCl	0.322	1.22	6.16	None
3	10.0	0.35		1.22	3.49	0.200	Positive
4	10.0	0.70		1.40	3.36	0.025	None-glucose depleted
			7X Dissolved O_2	1.50	3.06	0.029	Slightly more cells, less ethanol
			Pulse glucose	1.70			Positive, transient
5	10.0	0.1	3X H_3PO_4	0.352	1.57	6.98	None with respect to Med 2
6	10.0	0.1	3X $CaCl_2 \cdot 2H_2O$	0.333	1.54	6.35	None
			Pulse $MgSO_4 \cdot 7H_2O$	0.340	1.34	7.04	None
			Pulse NaCl	0.344	1.23	6.27	None
7	10.0	0.1	2X all other base nutrients	0.336	1.36	6.44	None
			Pulse inositol & para-aminobenzoic acid	0.340	1.56	6.00	None
			Pulse pyridoxine & nicotinic acid	0.350	1.36	6.19	None
			Pulse thiamine & pantothenate	0.338	1.31	6.48	None
8	10.0	0.1	Biotin (4μg/L)	0.805	2.74	2.53	Positive, transient
				0.430	1.94	4.65	Positive, steady state
9	10.0	0.1	4X Biotin(16μg/L)	0.436	1.91	4.70	None

(1) Minimal base medium composition given in Table 6.1

(2) With respect to the previous steady state except as noted

(3) Pulses are direct injections of nutrients into the fermentor.
If not specified as pulses, the nutrients are added to the feed reservoir.

from approximately 0.1 microgram/liter as a component of yeast extract, the cell mass increased 2.4 times in the transient overshoot and 1.3 times for the steady state response. Therefore, biotin is the first limiting nutrient for the minimal base medium in Table 6.1. A further increase in biotin had no effect, indicating the saturation concentration had been reached at 4.0 micrograms/liter.

6.4 Optimization of Growth Factors

The objective of this set of experiments was to search for growth and ethanol limiting nutrients in addition to biotin, focusing mainly on the growth factors. The same minimal base medium as given in Table 6.1 was used again with a small quantity of yeast extract (0.1 g/L) to provide for possible trace requirements of exotic factors.

The conditions and procedures for this set of experiments are those given in section 6.2 except as follows. Bacterial contamination was inhibited by including 50 mg/L of Penicillin-G (potassium salt) and 50 mg/L of Ampicillin in all media. A different fermentor with a working volume of 890 ml was used. The inlet and outlet gases from the fermentor were analyzed for carbon dioxide, oxygen, and nitrogen. In addition to optical density measurements, dry cell masses were determined by filtering steady state culture samples through membrane filters and by drying at 70 ^{o}C. for 72 hours. The correlation in Figure 3.1 was derived from the data of these experiments and can be used to convert cell optical density to cell dry mass. Transient responses usually occurred within four to six hours after pulse injections. Twenty four hours was allowed between injections for the nutrient to be washed out. Steady states after increasing feed reservoir concentrations were considered attained

when two consecutive samples about twelve hours apart were similar with respect to optical density and/or ethanol concentration. The dilution rate was 0.27/hr except as noted.

The transient responses are given in Table 6.3, which shows the steady state fermentor conditions before injection and the transient conditions after the specified times. Those components showing no transient response are noted in Table 6.4. Minimal in these tables refers to the minimal base medium in Table 6.1.

The carbon balance ratio column in Table 6.3 is a measure of the accuracy of the data. It is the ratio of carbon consumed, as measured by the decrease in glucose, to the carbon produced, as measured by the production of ethanol, cell mass, and carbon dioxide. A ratio of one indicates the data for the above components are consistent with respect to a carbon balance. Ratios less than or greater than one indicate the reported glucose consumption is, respectively, greater than or less than that accounted for by the reported ethanol, cell mass, and carbon dioxide production. A breakdown of the carbon balance data is given in Table 6.5.

As shown in Table 6.3, the minimal medium without additional growth factors resulted in low cell and ethanol productivities with only about one third of the available glucose consumed. Biotin added to this medium gave a very positive transient response. The steady state response to the addition of 4 micrograms/liter biotin was an approximate three fold increase in both cell and ethanol productivities.

Pantothenic acid was the next growth factor to show a positive

Table 6.3

Low Glucose Continuous Culture - Optimization of Growth Factor

Positive Transient Responses

Feed Media[1] (Before Injection)	Injections[2]	Time (hr)	Cell Mass (g/L)	Glucose Consumed (g/L)	Ethanol Produced (g/L)	Carbon Balance Ratio
1 x minimal base, 10 g/L Glucose & 0.1 g/L yeast extract			0.393	3.42	1.38	0.978
	Biotin (4.0 µg/L) (Feed Bottle)	21.5	0.696	8.06	3.09	1.07
1 x (minimal & Biotin) 10g/L Glucose & 0.1 g/L yeast extract			0.834	8.69	3.52	0.973
	Ca-Pantothenate (2.5 mg/L) (Fermentor)	4.0	1.08	9.42	4.04	1.05
1 x (minimal & Biotin & Panto) 15g/L Glucose & 0.1 g/L yeast extract			1.23	9.54	3.74	0.979
	Pyridoxine (1.25 mg/L) (Feed Bottle)	13.75	1.34	11.0	4.19	1.05
1 x (minimal & Biotin & Panto. & Pyr) 15 g/L Glucose & 0.1 g/L yeast extract			1.31	11.6	4.28	0.935
	Thia (0.133mg/L) Inos.(3.45mg/L) Nico (1.12mg/L) p-ABA(0.0518mg/L) (Fermentor)	6.75	1.38	13.2	4.73	0.888
1 x (minimal & Bio & Panto. & Pyr) 15g/L Glucose & 0.1 g/L yeast extract			1.33	12.3	4.49	0.908
	Thia (0.266mg/L) (Fermentor)	7.25	1.35	12.3	4.91	0.977
1 x (minimal & Bio & Panto & Pyr & Thia) 15g/L Glucose & 0.1 g/L yeast extract			1.48	12.8	4.82	0.941

Table 6.3

Continued

Positive Transient Responses

Feed Media (Before Injection)	Injections	Time (hr)	Cell Mass (g/L)	Glucose Consumed (g/L)	Ethanol Produced (g/L)	Carbon Balance Ratio
2/3 x (Minimal & Bio & Panto & Pyr & Thia) 10g/L Glucose & 0.1 g/L yeast extract			0.924	8.67	3.56	0.992
	Mineral Solution (2 x minimal) (Fermentor)	6.0	1.05	10.2	3.80	0.914
2/3 x (Minimal & Bio & Panto & Pyr & Thia) 10g/L Glucose & 0.1 g/L yeast extract			0.916	8.90	3.41	0.938
	$(NH_4)_2SO_4$ (1.12 g/L) (Fermentor)	10.75	0.989	9.74	3.56	0.898
1 x $(NH_4)_2SO_4$ 2/3 x (other minimal comp. & Bio & Panto & Pyr & Thia) 10g/L Glucose & 0.1 g/L yeast extract			1.26	9.98	3.74	0.954

(1) Growth Factors (Abbreviations)

	Feed Concentration (mg/L) after Injection
Biotin (Bio)	0.004
Pantothenic Acid (Panto)	1.25
Pyridoxine (Pyr)	1.25
Thiamine (Thia)	0.133
Inositol (Inos)	
Nicotinic Acid (Nico)	
P- Aminobenzoic Acid (p-ABA)	

(2) Concentrations given with (feed bottle) are for injected nutrients including the feed. Concentrations given with (fermentor) are for injected nutrients and are incremental to the feed.

Table 6.4

Low Glucose Continuous Culture - Optimization of Growth Factors

Absence of Transient Response

Media	Injection	Probable Reason for Non Response
(1X) (Minimal & Biotin & Pantothenic Acid) 10g/L Glucose & 0.1 g/L yeast extract	Pyridoxine	Glucose was depleted
(1X)(Minimal & Bio. & Pant. & Pyr. & Thia.) 15g/L Glucose & 0.1 g/L yeast extract	Inositol, P-ABA, Nicotinic Acid	Growth limiting component not injected
(1X) (Minimal & Bio & Pant & Pyr & Thia) 15g/L Glucose & 0.1 g/L yeast extract	Yeast Extract	Growth limiting component not in yeast extract
(2/3X) (Minimal & Bio.& Pant. & Pyr. & Thia) 10g/L Glucose & 0.1 g/L yeast extract	Trace Elements	Growth limiting component not in trace elements solution
(2/3X) (Minimal & Bio. & Pant. & Pyr. & Thia) 10g/L Glucose & 0.1 g/L yeast extract	Biotin, Pantothenic Acid	Still in excess, not limiting component

Table 6.5

Carbon Balance

for Steady State Low Glucose Continuous Culture - Optimization of Growth Factors

Media	Dilution Rate(hr^{-1})	Glucose Feed(g/L)	Carbon Produced (g/L) inEthanol	inCO$_2$	inCells	Total	Carbon Consumed(g/L)	Carbon Production/ Consumption Ratio
(1X) Minimal	0.27	10.1	0.720	0.428	0.196	1.34	1.37	0.978
(1X) (Minimal & Biotin)	0.27	9.84	1.84	1.13	0.417	3.39	3.48	0.973
(1X) (Minimal & Biotin & Pantothenic Acid)	0.27	9.84	2.11	1.14	0.605	3.85	3.85	1.00
(1X) (Minimal & Biotin & Pantothenic Acid)	0.345	9.89	1.24	0.924	0.385	2.55	2.67	0.954
(1X) (Minimal & Biotin & Pantothenic Acid)	0.42	10.0	0.631	0.432	0.179	1.24	1.26	0.984
(1X) (Minimal & Biotin & Pantothenic Acid)	0.27	14.2	1.95	1.18	0.615	3.74	3.82	0.979
(1X) (Minimal & Biotin & Pantothenic Acid & Pyridoxine)	0.27	14.9	2.23	1.46	0.653	4.34	4.64	0.935
(1X) (Minimal & Biotin & Pantothenic Acid & Pyridoxine & Thiamine)	0.27	14.9	2.51	1.55	0.740	4.80	5.12	0.941
(2/3X) (Minimal & Biotin & Pantothenic Acid & Pyridoxine & Thiamine)	0.27	10.24	1.86	1.11	0.462	3.44	3.47	0.992

response after the biotin requirement was met. Previously, it was demonstrated that unless this biotin requirement was first met, pantothenic acid and pyridoxine did not show positive responses, implying biotin is needed for an important reaction before pantothenic acid or pyridoxine can be utilized in following reactions. Lichstein (3) cited evidence from Williams et al. (4) and from Strauss and Moat (5) that biotin stimulated the hexokinase catalyzed conversion of glucose to glucose 6-phosphate. This is the first step of glycolysis and precedes reactions utilizing Ca-pantothenate and pyridoxine as seen in Figure 7.2. The pulse addition of pantothenic acid to the fermentor increased ethanol and cell production to the point where the glucose was almost entirely consumed such that it then became the limiting substrate. The glucose concentration was then increased to 15 g/L for the steady state pantothenic acid response and for the next growth limiting nutrient, pyridoxine.

Pyridoxine gave positive transient and steady state responses. Next a combination of thiamine, inositol, nicotinic acid, and p-aminobenzoic acid was pulsed into the fermentor and yielded a positive ethanol production response. After steady state was re-established, pulsing just thiamine also produced a positive response in ethanol production. The steady state response to thiamine addition to the feed showed a slight increase in cell mass production and confirmed the positive pulse effect on ethanol production. Subsequent injections of inositol, nicotinic acid and p-aminobenzoic acid to the fermentor showed no response (Table 6.4). Yeast extract also had no further effect at this point with the minimal base medium supplemented by biotin, pantothenic acid, pyridoxine, and thiamine.

To see the growth limiting concentrations for the medium minerals, the medium was then diluted to give 10 g/L glucose and only 2/3 the minimal base and previous growth factor concentrations. The absence of a response to biotin and pantothenic acid confirmed they were still in excess. An injection of a mineral mixture gave a positive response. A subsequent injection of $(NH_4)_2SO_4$ also gave a positive response, indicating it was the limiting component that was responsible for the earlier positive response with the mineral mixture. The steady state response for $(NH_4)_2SO_4$ addition to the feed showed a significant cell increase but a smaller ethanol increase. With this feed all sugars were utilized and the search for new limiting substrates ended.

The steady state continuous culture responses to medium composition shifts are given in Table 6.6. From this table the incremental effects of adding biotin, pantothenate, pyridoxine, thiamine and ammonium sulfate can be determined. The effects of varying dilution rates for the biotin and pantothenate supplemented medium are also presented. Consistent with growth associated ethanol production, the highest specific ethanol productivity, 1.42 g-ethanol/g-cell-hr, occurred with biotin and pantothenic acid additions at the highest dilution rate utilized, 0.42/hr.

The highest ethanol yield, 0.42 g-ethanol/g-glucose consumed, was achieved with the same medium but at 0.27/hr dilution rate. Combined with a high cell yield of 0.126 g-dry cells/ g-glucose consumed, practically the entire 10 g/L glucose feed was consumed at these conditions with only biotin and pantothenic acid supplemented medium. For 15 g/L glucose feed, pyridoxine and thiamine were also required for complete

Table 6.6

Low Glucose Continuous Culture - Optimization of Growth Factors

Steady State Glucose, Cell Mass, and Ethanol Data

Media	Feed Glucose	Dilution Rate	Glucose Residue	Glucose Utilized	Dry Cell Mass	Ethanol	$Y_{x/s}$ (1)	$Y_{p/s}$ (2)	q_p (3)
	(g/L)	(hr^{-1})	(g/L)	(g/L)	(g/L)	(g/L)			(hr^{-1})
Minimal	10.1	0.27	6.68	3.42	0.393	1.38	0.115	0.404	0.948
Minimal & Biotin	10.0	0.27	1.31	8.69	0.834	3.52	0.0960	0.405	1.14
Minimal & Biotin & Pantothenate	9.84	0.27	0.22	9.62	1.21	4.04	0.126	0.420	0.901
Same as above	9.89	0.345	3.21	6.68	0.770	2.38	0.115	0.356	1.07
Same as above	10.0	0.42	6.84	3.16	0.358	1.21	0.113	0.383	1.42
Same as above	15.0	0.27	5.46	9.54	1.23	3.74	0.129	0.392	0.821
Minimal & Biotin & Pantothenate & Pyridoxine	15.0	0.27	3.40	11.6	1.31	4.28	0.113	0.369	0.882
Minimal & Biotin & Pantothenate & Pyridoxine & Thiamine	15.0	0.27	2.20	12.8	1.48	4.82	0.116	0.377	0.879
(2/3X)(Minimal & Biotin & Pantothenate & pyridoxine & thiamine	10.2	0.27	1.53	8.67	0.924	3.56	0.107	0.411	1.04
(1X) $(NH_4)_2SO_4$, (2/3X)(other minerals & Biotin & Pantothenate & Pyridoxine & Thiamine)	10.0	0.27	0.02	9.98	1.26	3.74	0.126	0.375	0.801

(1) $Y_{x/s}$ = g-cells /g-glucose consumed

(2) $Y_{p/s}$ = g-ethanol /g-glucose consumed

(3) q_p = g-ethanol /g-cells /hr

glucose consumption at 0.27/hr dilution rate.

The cell yields for low glucose concentration feeds of about 10 g/L in continuous cultures ranged from 0.10 to 0.13 g-cells/ g-glucose consumed. These yields were significantly higher than those obtained with higher glucose concentration feeds in the order of 50 - 100 g/L. The higher cell yields probably resulted in part from lower ethanol inhibition with lower glucose feeds. High cell yields, however, are not generally desirable for ethanol production because they result in lower ethanol yields and lower ethanol specific productivity. Furthermore, cell recycle can partly compensate for lower cell yields.

Having achieved almost total glucose utilization, attention was focused on increasing the ethanol yield, the most sensitive parameter of ethanol cost. One approach is to decrease those nutrients required more for growth than for ethanol production. The requirements and thus sensitivity of each of the known limiting nutrients for growth and ethanol production are given in Table 6.7. These requirements are defined as grams cell mass, glucose consumed, or ethanol produced per gram of limiting nutrient available. They approximate yield coefficients as the available limiting nutrient approaches total consumption. For this case, a balanced minimal medium composition for any desired level of cell mass or ethanol can be determined from the table. Therefore, costly excess concentrations of all other substrates can also be reduced as glucose is made the limiting substrate for practical processes.

To determine if the ratio of ethanol to cell mass production can be changed by medium composition as predicted by the Table 6.7 sensitivities, the effect of varying the concentration of each component as the

Table 6.7

Limiting Nutrient Requirements for Growth and Ethanol Production

Media	Limiting Nutrient	g-Dry cells/ g-limiting Nutrient	g-Glucose used/ g-limiting Nutrient	g-Ethanol/ g-limiting Nutrient
(1x) (Minimal) 10 g/L Glucose	Biotin	3.9×10^6	3.4×10^7	1.4×10^7
(1x) (Minimal & Biotin) 10 g/L Glucose	Panthothenate	8.3×10^4	8.6×10^5	3.5×10^5
(1x) (Minimal & Bio & Pant) 15 g/L Glucose	Pyridoxine	6.0×10^5	4.8×10^6	2.0×10^6
(1x) (Minimal & Bio & Pant & Pyr) 15 g/L Glucose	Thiamine	4.4×10^6	3.9×10^7	1.4×10^7
(1x) (Minimal & Bio & Pant & Pyr & Thia) 15 g/L Glucose	$(NH_4)_2SO_4$	2.94	27.6	11.3
(2/3x) (Minimal & Bio & Pant & Pyr & Thia) 10 g/L Glucose	$(NH_4)_2SO_4$	2.91	28.3	10.8

limiting nutrient in continuous culture should be established. Furthermore, the optimal level of each limiting nutrient with respect to various yields and productivities can be determined from this study as will be seen in the next chapter.

6.5 Conclusions

Pulse injections of individual nutrients for transient responses and shifts in feed medium composition for steady state responses in continuous culture provide an effective method to identify and approximately determine media requirements for growth and ethanol production.

The limiting nutrients in the minimal synthetic medium of Table 6.1 are present in yeast extract. Biotin was established as the first limiting nutrient of yeast extract; its requirement must be satisfied before the limiting effects of other requirements can be observed. The most important of these other requirements were determined to be pantothenate, pyridoxine, thiamine and ammonium sulfate.

The requirements of the limiting nutrients per unit cell mass produced, ethanol produced and glucose consumed can be calculated at limiting concentrations, as shown in Table 6.7, to provide approximate bases for scale-up to higher concentration media.

6.6 References for Chapter 6

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Chapter 7

7. Media Requirements for 100 g/L Glucose Continuous Cultures

7.1 Scale-up of Low Glucose Medium

In Chapter 6, 10 g/L glucose feeds supplemented with 0.1 g/L yeast extract and synthetic vitamins were shown to be totally utilizable in continuous cultures at a dilution rate of 0.27/hr. In this chapter the continuous culture media requirements for 100 g/L glucose feeds are to be established. The scale-up from 10 to 100 g/L glucose feeds with approximately the same relative concentrations of secondary (non glucose) nutrients will be described first.

The procedure and fermentor set-up for this set of experiments were essentially the same as those for the preliminary media optimization studies described in Chapter 6. The compositions of the feed media (Medium 27-Medium 33) are listed in Table 7.1. This set of experiments was run after low concentration (10-15 g/L) glucose feeds showed that biotin, pantothenate and pyridoxine were required growth factors as reported in Wilke et al (1). Hence, these were the growth factors in the starting and minimal feed medium, Medium 27. The concentration of these growth factors and of all the minerals except ammonium sulfate were scaled-up by an approximate factor of five relative to their concentrations for 10 g/L glucose feed. Even though the glucose feed concentration was scaled-up by a factor of ten, these other component concentrations were scaled-up less so they would be relatively deficient to allow positive responses to pulse and step changes in their concentrations. Except for ammonium sulfate, the minerals were in the same

Table 7.1

Media for Continuous Cultures with 100 g/l Glucose Feed

	Units	Med27	Med28	Med29	Med30	Med31	Med32	Med33
Glucose	g/l	100	100	100	100	100	100	100
$(NH_4)_2SO_4$	g/l	3.54	3.54	3.54	3.54	4.72	4.72	3.80
$CaCl_2 \cdot 2H_2O$	g/l	0.059	0.059	0.059	0.059	0.118	0.118	0.0723
$MgSO_4 \cdot 7H_2O$	g/l	0.248	0.248	0.248	0.248	0.496	0.496	0.304
K_2HPO_4	g/l	0.730	0.730	0.730	0.730	1.46	1.46	0.894
Yeast extract	g/l	1.00	1.00	1.00	1.00	1.00	1.00	2.00
Trace element (see Table 6.1)	ml/l	2.50	2.50	2.50	2.50	5.00	5.00	3.06
EDTA	g/l	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Biotin	μg/l	26.9	53.8	51.8	51.1	26.9	53.8	33.0
Pantothenate	mg/l	6.92	6.92	6.92	6.93	6.92	13.8	8.48
Pyridoxine	mg/l	6.66	6.66	6.66	6.66	6.66	13.3	8.15
P-aba	mg/l				0.31		0.517	0.116
Thiamine	mg/l				0.832		1.38	0.310
Nicotinic acid	mg/l				6.67		11.1	2.50
Inositol	mg/l				20.7		34.55	7.77

ratios as those in Table 6.1 for 10 g/L glucose continuous cultures. The ammonium sulfate concentration was scaled-up 7.5 times that in Table 6.1. The air sparge was maintained at about the same rate, about 40 ml/min, as that used in the low glucose medium cultures.

The scaled-up media in Table 7.1 differed from the lower concentration media also in having ethylenediamine tetra-acetic acid (EDTA) present. EDTA served as a chelating agent mainly to prevent precipitation of calcium, magnesium and iron with phosphates. Precipitation was naturally a greater problem with higher concentration media. Another difference between the media in Tables 6.1 and 7.1 was that potassium and phosphates were provided separately as KCl and H_3PO_4 in the low concentration media but were together as KH_2PO_4 in the initial high concentration media and K_2HPO_4 in the later high concentration media. Rothstein (2) reported that the monovalent anion $H_2PO_4^{-1}$, but not the bivalent anion HPO_4^{-2} was absorbed by yeast. Nevertheless, the form of the ion sources was assumed not important because their concentrations in solution should have been approximately the same as determined by the same dissociation equilibrium and pH.

Table 7.2 summarizes the results for continuous cultures with the 100 g/L glucose feed media. The initial steady state with the minimal medium (Medium 27) at a dilution rate of 0.19 hr^{-1} resulted in 53.2% glucose utilization. Raising the dilution rate to 0.28 hr^{-1}, which was approximately the dilution rate for the 10 g/L glucose cases, decreased glucose utilization to 22.3%. Supplementing the minimal medium with additional biotin (Medium 28) in the steady state feed or with pulses of pyridoxine, pantothenate, and biotin (Medium 29) did not significantly

Table 7.2

Continuous Culture with 100 g/l Glucose Feed

Feed	Medium Number	Medium [Pulse]	Dilution Rate (hr^{-1})	Dry Cell Mass (g/l)	Ethanol (g/l)	Residual Sugar (g/l)
Steady State	(27)	Minimal	0.19	3.54	18.0	46.8
Steady State	(27)	Minimal	0.28	1.86	8.92	77.7
Steady State	(28)	Minimal + Biotin	0.28	2.30*	8.91	75.8
Pulse	(29)	Above Medium + [Pyridoxine + Pantothenate + Biotin]	0.28	2.24*	8.92	76.0
Steady State	(30)	Minimal + Biotin + p-amino benzoic acid + Thiamine + Nicotinic acid + Inositol	0.28	2.77*	11.5	69.2
Pulse	(30)	Above Medium + $[(NH_4)_2SO_4]$	0.28	2.87*	12.3	64.8
Steady State	(31)	Minimal + Minerals	0.28	2.64*	12.3	66.0
Steady State	(32)	Minimal + Minerals + Vitamins	0.28	3.86	16.7	52.2

* Determined by Beckman spectrophotometer; unstarred dry cell mass determined by Bausch and Lomb spectrophotometer

change the glucose consumption. Although these three vitamins were shown to be required in the low glucose feed cases, they were apparently in sufficient quantity in the minimal medium. It was then shown that the minimal medium was deficient in some combination of p-aminobenzoic acid, thiamine, nicotinic acid, and myo-inositol since supplementing the minimal medium with this combination (Medium 30) significantly increased cell mass and ethanol production and sugar utilization. Supplementing the minimal medium with additional minerals (Media 30 and 31) also improved sugar utilization significantly. The highest productivity medium combined supplemental vitamins and minerals (Medium 32) to produce 47.8% sugar utilization. The vitamin content of this medium was higher than that for the vitamins supplemented Medium 30, so the extent of synergism with respect to vitamins and minerals was not clear.

Even with the best medium, the sugar utilization was low relative to that obtained with high yeast extract concentration medium by Cysewski (3). Possible reasons included substrate deficiencies, ethanol inhibition, substrate inhibition, non-optimal oxygen tension and changes in the organism. Organism stability effects are discussed later in this chapter; the other effects were environmental and are investigated in a later chapter.

7.2 Continuous Cultures with High Yeast Extract Medium

To determine if nutrient deficiencies were responsible for the relatively low cell and ethanol productivities from the media in Table 7.1, the high yeast extract medium used by Cysewski (3) (Table 1.1) was run in continuous culture. This medium contained 8.5 g/L yeast extract per 100 g/L glucose. The fermentation conditions for running this

medium were approximately the same as for the Table 7.1 media except as noted.

The results for the high yeast extract medium are given in Table 7.3 for both this work and that of Cysewski (3). Cysewski used only 89 g/L glucose in the feed compared to 100 g/L in this work, but obtained more than twice the ethanol and cell mass of this work for both 0.27/hr and 0.14/hr dilution rates. The air sparge rate between about 20 to 30 ml/min had no effect in this work. Based on later work, the increased productivities of cells and ethanol with the air sparge rate at about 10 ml/min were probably not due to the air sparge effect but to the simultaneous decrease in dilution rate. The air sparge rate was decreased to more closely simulate Cysewski's very low dissolved oxygen tension of 0.07 mm Hg oxygen. Matching the dissolved oxygen tension, however, was not possible because of the lack of measurement sensitivity.

The much lower cell and ethanol productivities of this work with respect to those of Cysewski with the same medium indicated differences in the environmental conditions and/or the organism. Cysewski (3) reported that the effect of dissolved oxygen tension was especially important. The increase in ethanol and cell mass productivities with high yeast extract medium relative to the highest productivity medium (Medium 32) derived from scale-up of low glucose medium indicated deficiencies in the scaled-up medium.

7.3 Higher Productivity Continuous Cultures

Further media optimization was combined with optimization of the dissolved oxygen and carbon dioxide concentrations to increase the

Table 7.3

Continuous Culture with Cysewski's Medium

Investigator	Dilution Rate (hr^{-1})	Air Sparge Rate (ml/min)	Feed Glucose (g/l)	Ethanol (g/l)	Dry Cell Mass (g/l)
This Work	0.27	~30	100	19.8	4.2
This Work	0.27	~20	100	19.1	4.2
This Work	0.14	~10	100	34.5	6.7
Cysewski(2)	0.27		89	32	9.5
Cysewski(2)	0.14		89	38	14.0

ethanol productivity. The pulse injection followed by step feed change technique described in Chapter 6 was used again, starting with the minimal medium (Medium 27) in Table 7.1. The outcome of this set of experiments carried out approximately over six months was the medium and corresponding results given in Table 7.4. Although this medium did not change greatly from the starting medium, its productivity did increase significantly from the initial scale-up of low glucose medium reported in Table 7.2 and from the high yeast extract medium.

At least three factors were involved in the increased concentrations from 18.0 g/L to 35.5 g/L for ethanol and from 3.54 g/L to 7.95 g/L for cell mass at about 0.19-0.20/hr dilution rate. First, the oxygen and carbon dioxide concentrations were made more favorable. Second, the starting medium was improved by adding thiamine and myo-inositol. Third, there appeared to be an adaptation or mutation of the organism to produce more cell mass and ethanol from a medium which was entirely synthetic except for 1.0 g/L yeast extract.

This last effect was the result of natural selection inherent in long term continuous cultures. The increased ethanol productivity was in part due to increased ethanol tolerance as demonstrated in the chapter on environmental effects. Another apparent effect was an adaptation to less complex media accompanied by reduced requirements for complex and pre-formed nutrients. The reduced need for these components indicated development of additional biosynthetic capabilities for their production within the cell.

Koser (4) reported that reducing or eliminating the supply of a previously essential vitamin gradually eliminated the need for that

Table 7.4

High Productivity Continuous Culture

Medium

Glucose	100.0	g/l
$(NH_4)_2SO_4$	3.54	g/l
KCl	0.625	g/l
H_3PO_4	0.411	g/l
$MgSO_4 \cdot 7H_2O$	0.248	g/l
$CaCl_2 \cdot 2H_2O$	0.059	g/l
Yeast extract	1.0	g/l
Trace elements solution (see Table 6.1)	2.5	ml/l
Biotin	0.0269	mg/l
Pantothenate	6.92	mg/l
Pyridoxine	6.66	mg/l
Inositol	20.0	mg/l
Thiamine	1.34	mg/l
Penicillin G	0.314	g/l
Ampicillin	0.0375	g/l
Streptomycin	0.010	g/l

Fermentation Parameters

Dilution Rate	0.205	hr^{-1}
Glucose Feed Concentration	96.9	g/l
Dry Cell Mass	7.95	g/l
Ethanol	35.5	g/l
Residual Glucose	10.5	g/l
Cell Mass Yield	0.092	
Ethanol Yield	0.411	
Specific Ethanol Productivity	0.915 g etoh/g cell-hr	

vitamin for growth in a number of different microorganisms. Leonian and Lilly (5) successively subcultured several strains of Saccharomyces cerevisiae in chemically well defined media deficient in one or more of five vitamins normally needed for fast growth. They reported formation of strains able to grow readily without some or in two cases all of the previous requirements.

Figure 7.1 shows the ethanol concentrations produced by older, less adapted yeast with varying yeast extract concentrations and by newer, more adapted yeast with varying corn steep liquor concentrations and a single yeast extract concentration. Corn steep liquor should be inferior to yeast extract as a source of complex factors for growth and ethanol production. However, the adapted yeast given corn steep liquor produced more ethanol and cell mass than the unadapted yeast given the same concentration yeast extract.

With 1.0 g/L yeast extract, biotin, Ca-pantothenate and pyridoxine in the feed, the unadapted yeast produced about 18 g/L ethanol compared to 28 g/L (not shown in Figure 7.1) by the adapted yeast. Additions of thiamine and inositol increased the ethanol concentration to about 33 g/L for the adapted yeast. The corn steep liquor media for adapted yeast was also supplemented by the five growth factors. Therefore, the differences in productivities between adapted yeast with corn steep liquor and unadapted yeast with yeast extract may be partly due to thiamine and myo-inositol.

An important point in Figure 7.1 was the 28 g/L ethanol produced by the adapted yeast with no yeast extract or corn steep liquor, i.e., with a completely synthetic medium. Extrapolating the unadapted yeast

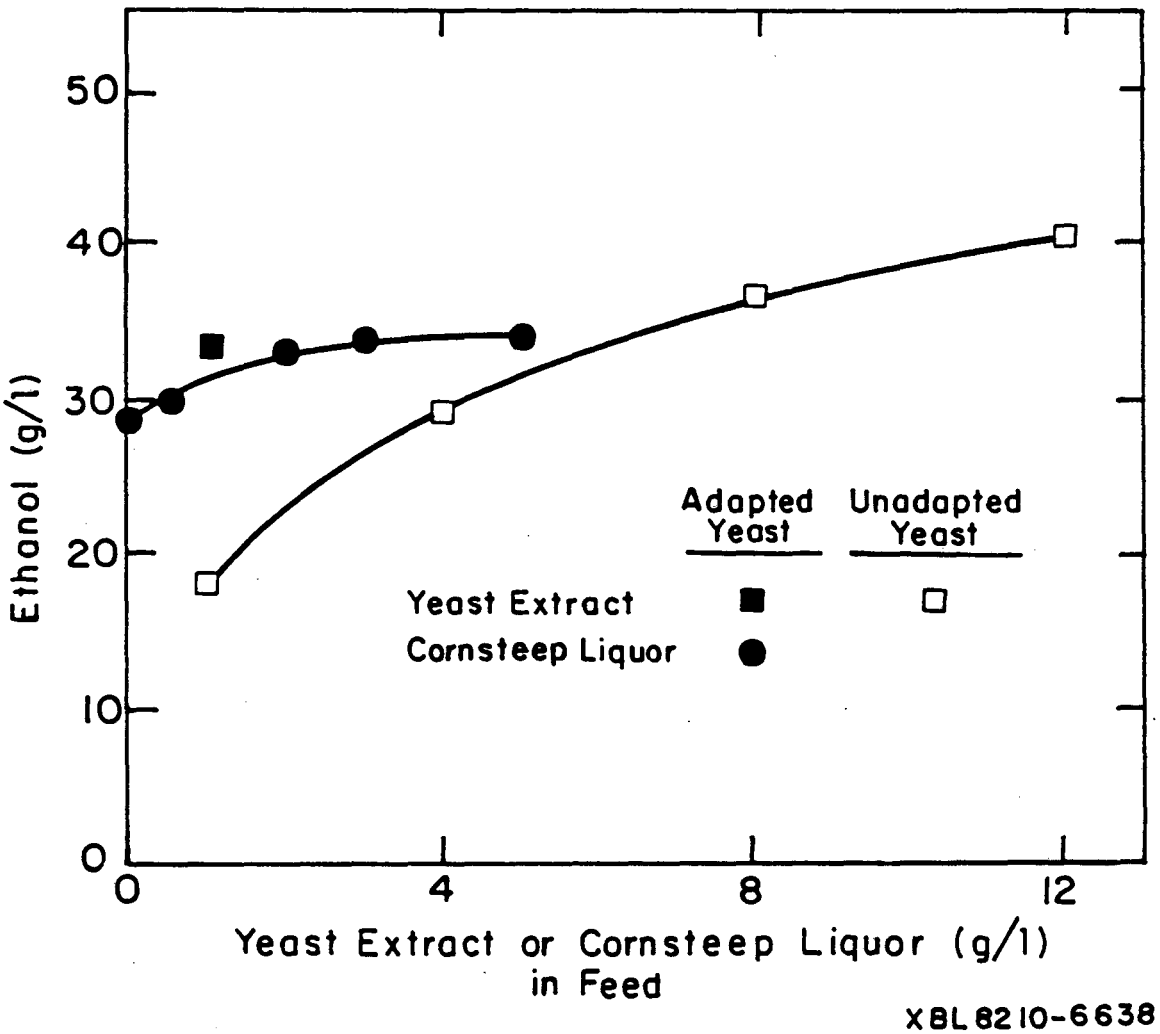

Figure 7.1 Effect of Yeast Extract and Cornsteep Liquor Concentration on Ethanol Production before and after Probable Adaptation to Minimal Media with Minimum Complex Factors

ethanol concentrations to zero concentration indicated a much lower ethanol concentration for unadapted yeast. Previous experiments with unadapted yeast in media supplemented with all five growth factors also produced much less than 28 g/L ethanol.

At this stage of development, the adapted yeast can synthesize all needed growth factors, including previously unknown factors. Nevertheless, addition of catalytic growth factors can still be stimulatory to growth or ethanol production rates if other factors are not limiting. Even though these growth factors are no longer "required" in the strict sense of essential, they may still be referred to as "required" in the stimulatory sense.

7.4 Individual Nutrient Effects

Having established a base medium with a reasonable ethanol productivity, the effect of feed concentration of the individual nutrients was studied. The sensitivity of the major fermentation parameters was determined with respect to the major inorganic ions and growth factors likely to be required in industrial feed media. Trace elements, sulphates and chlorides were not considered. Using these data, media can be formulated to optimize any of these parameters. The parameters chosen depend on the process. Furthermore, these data are useful to obtaining a conceptual understanding of the functions of these nutrients.

The media compositions for the individual nutrient studies are listed in Table 7.5 Although the effect of only one nutrient was to be determined in each set of experiments, in certain cases second or third

Table 7.5

Media for Studying Individual Nutrient Effects

		Individual Nutrient Studied											
	Units	$(NH_4)_2SO_4$	$MgSO_4 \cdot 7H_2O$	$CaCl_2$	Biotin	PO^{-3}_4	Panto-thenate	KCl	Pyrido-xine	Thiamine	Inositol	CSL	Yeast ext
$(NH_4)_2SO_4$	g/l	0.885-6.20	3.54	3.54	3.54	3.54	2.66-3.54	3.54	2.66	3.54	3.54	3.54	3.54-4.425
KCl	g/l	0.208-0.625	0.625	0.625	0.625	0.625	0.625	0.0368-0.625	0.625	0.208-0.625	0.208	0.625	0.625
H_3PO_4	g/l	0.411	0.411	0.411	0.411	0.411-0.738	0.411	0.411	0.411	0.411	0.411	0.411	0.411
$MgSO_4 \cdot 7H_2O$	g/l	0.124	0.124-0.331	0.124-0.248	0.124-0.248	0.248	0.124	0.124	0.124	0.124	0.124	0.124	0.248
$CaCl_2 \cdot 2H_2O$	g/l	0.0295	0.060	0.0295-0.060	0.03-0.06	0.059	0.03	0.0295	0.03	0.03	0.03	0.0295	0.059
Yeast extract	g/l	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0-19.4
Trace elements	ml/l	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Biotin	μg/l	5.27	26.9	26.9	2.72-56.2	26.9	5.1-5.27	5.27	5.27	5.27	5.27	5.26	26.9
Pantothenate	mg/l	2.5-6.92	6.92	6.92	6.92	6.92	0.063-6.92	2.5	2.0	2.5	2.5	2.0	6.92
Pyridoxine	mg/l	3.0-6.67	6.67	6.66	6.66	6.66	3.33-6.66	3.0	0-6.66	3.0	3.0	1.0	6.66
Thiamine	mg/l	1.34	1.34	1.34	1.34	1.34	1.34	0.335-2.0	1.34	0 - 8.0	0.34-1.34	1.34	
Inositol	mg/l	10	10	10	10	10-20	10	10	10	10	0-20	1.25	
$Na_3PO_4 \cdot 12H_2O$	g/l		0.925	0.925	0.65-0.925		0.65						
$Na_2HPO_4 \cdot 7H_2O$	g/l	0.34					0.34	0.34	0.34	0.34	0.34		
Penicillin G	g/l	0.30	0.29	0.29	0.-0.29	0.26-0.31	0.-0.29	0.30	0.30	0.30	0.32	0.3	
Ampicillin	g/l	0.0375	0.0375	0.0375	0.-0.0375	0.0375	0.-0.0375	0.0375	0.0375	0.0375	0.05	0.0375	
Streptomycin	g/l	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	
K_2HPO_4	g/l												0.730
EDTA	g/l												0.25

component concentrations were also varied. In such cases, other experiments showed that the range of variation specified for the second or third components should have insignificant effect.

The fermentation conditions were approximately the same as for the high productivity continuous cultures in the previous section except as follow: dilution rate = 0.20/hr, fermentor working volume = 670 ml, air sparge rate = 20 ml/min, and nitrogen sparge rate = 100 ml/min. Except for the early study of the effect of yeast extract, adapted yeast was used for the individual nutrient studies.

7.4.1 Conceptual Model for Nutrient Effects

a) Transport of Nutrients

Transport of the nutrient from the medium into the cell is the first step toward its utilization. Before studying the effects of nutrient concentrations on reaction rates, the extent of transport limitations should be considered.

Transport kinetic parameters are reported in Table 7.6. Uptake of all these nutrients by Saccharomyces cerevisiae and closely related species, as specified, follow Michaelis-Menten kinetics with respect to the fermentor medium concentration. Saturation kinetics with specificity for substrates and sensitivity to low concentrations of inhibitors indicate the transport mechanism for these nutrients through the cell membrane is by saturable carriers or receptors. Because conditions were not well defined or reported for some of the kinetic studies, the Michaelis-Menten parameters given should be considered order of magnitude values. Nevertheless, they provide guidelines for determining mass

Table 7.6

Michaelis-Menten Parameters for Nutrient Transport into Yeasts
(*Saccharomyces cerevisiae*, except as noted)

Nutrient	K_m	V_{max}	Comment	Reference
NH_4^+	0.001 - 0.02 mM			Kleiner (6)
K^+	0.005 - 0.01 M	1000 mM/kg cells/hr		Rothstein and Bruce (7)
$H_2PO_4^-$	0.47 mM	54 mmole/kg cells/hr		Rothstein (2,42)
	0.0006 mM		sodium dependent mechanism	Roomans et al. (43)
	0.014 - 0.03 mM		sodium independent mechanism	Roomans et al. (43,44)
Mg^{+2}			Michaelis-Menten kinetics, active transport	Rothstein (2), Furhrmann(8)
Ca^{+2}	0.044 mM	0.066 nmole/min/10^8 cells	*Schizosaccharomyces pombe*	Boutry et al. (9)
SO_4^{-2}	0.01 mM	0.15 mmole/min/kg cells	pH = 4	Roomans et al. (45)
	0.06 mM	0.75 mmole/min/kg cells	pH = 6	Roomans et al. (45)
Biotin	8.0×10^{-7} M	2.0×10^{-7} moles /min/mg cell		Becker and Lichstein (10)
Pyridoxine	3.6×10^{-7} M		*Saccharomyces carlsbergensis*	Shane and Snell (11)
Thiamine	0.00018 mM			Iwashima et al. (12)

transfer limiting concentrations for many nutrients.

A second common characteristic of all the nutrients in Table 7.6 is the requirement for active transport. The need for energy in transporting these nutrients is evidenced by their transfer against high concentration gradients. For example, Rothstein (2) reported that K^+ ions could be transported into cells against a 1000 to 1 concentration gradient in exchange for H^+ ions transported out of the cell against a 100 to 1 gradient for a total gradient of 10^5 to 1 for the pair of ions. Suomalainen and Oura (38) reported influx of orthophosphate against a 100 to 1 gradient, and Okorokov et al (39) reported active transport of magnesium against a 110 to 1 gradient. Furthermore, resting cells are relatively impermeable to cations and anions by passive diffusion because their transport is linked to metabolism for energy.

b) Metabolism of Nutrients

Figures 7.2 and 7.3 summarize the generally accepted pathways from glucose to many of the major precursors of organic cell components as well as to ethanol for Saccharomyces cerevisiae under fermentative conditions. These figures also show the required growth factors and inorganic ions in captions for the pathways given; their functions are described more fully in Tables 7.7 and 7.8, respectively.

A general conceptual model is useful in explaining the effects of these individual growth factors and inorganic ions on cell growth and ethanol production in terms of the pathways of Figures 7.2 and 7.3. This model would, furthermore, be helpful in predicting the general effect of the feed concentration of each of the required medium

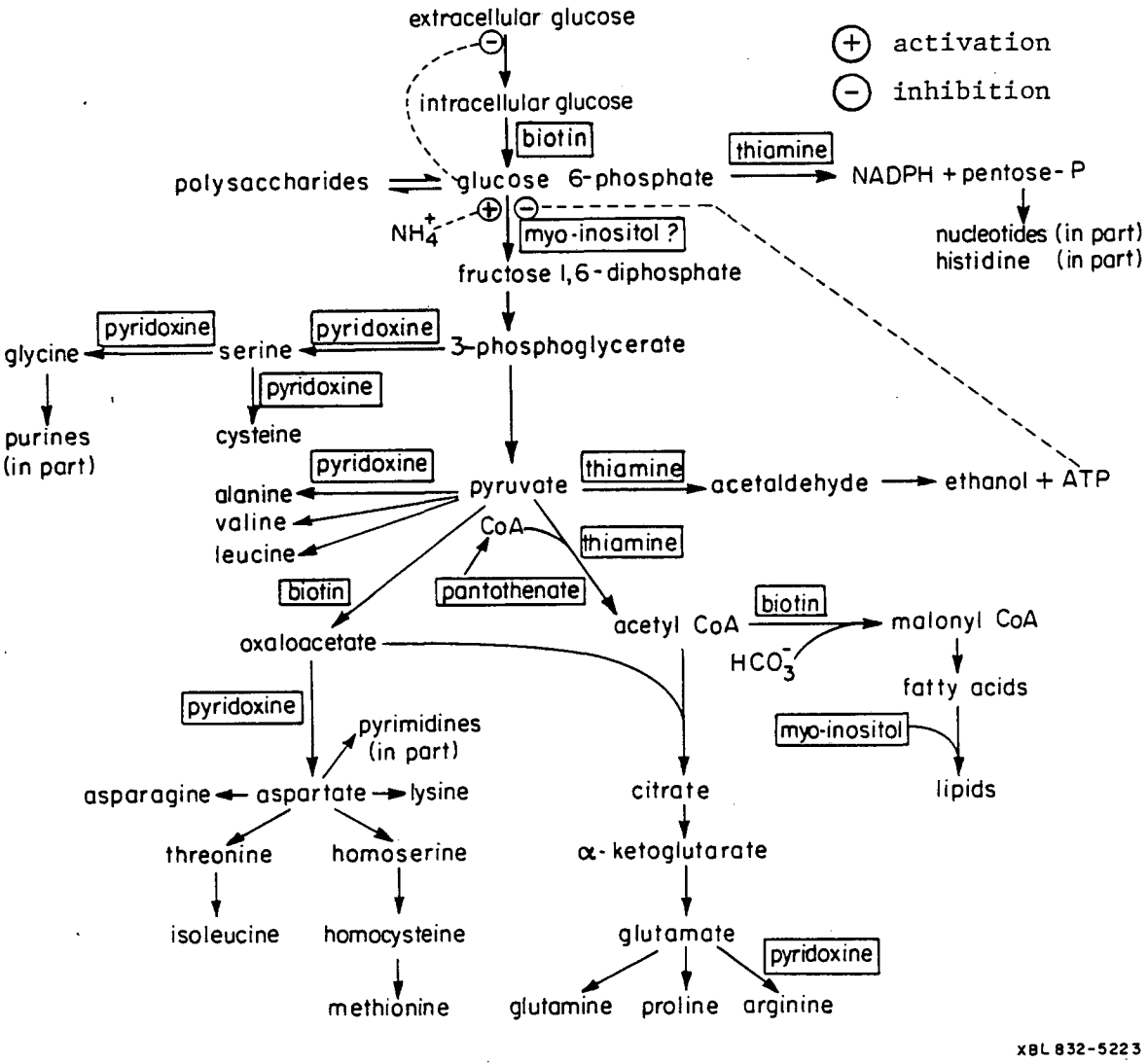

Figure 7.2 Growth Factors as Cofactors for Catalyzing Pathways to Production of Ethanol and Cell Mass Components

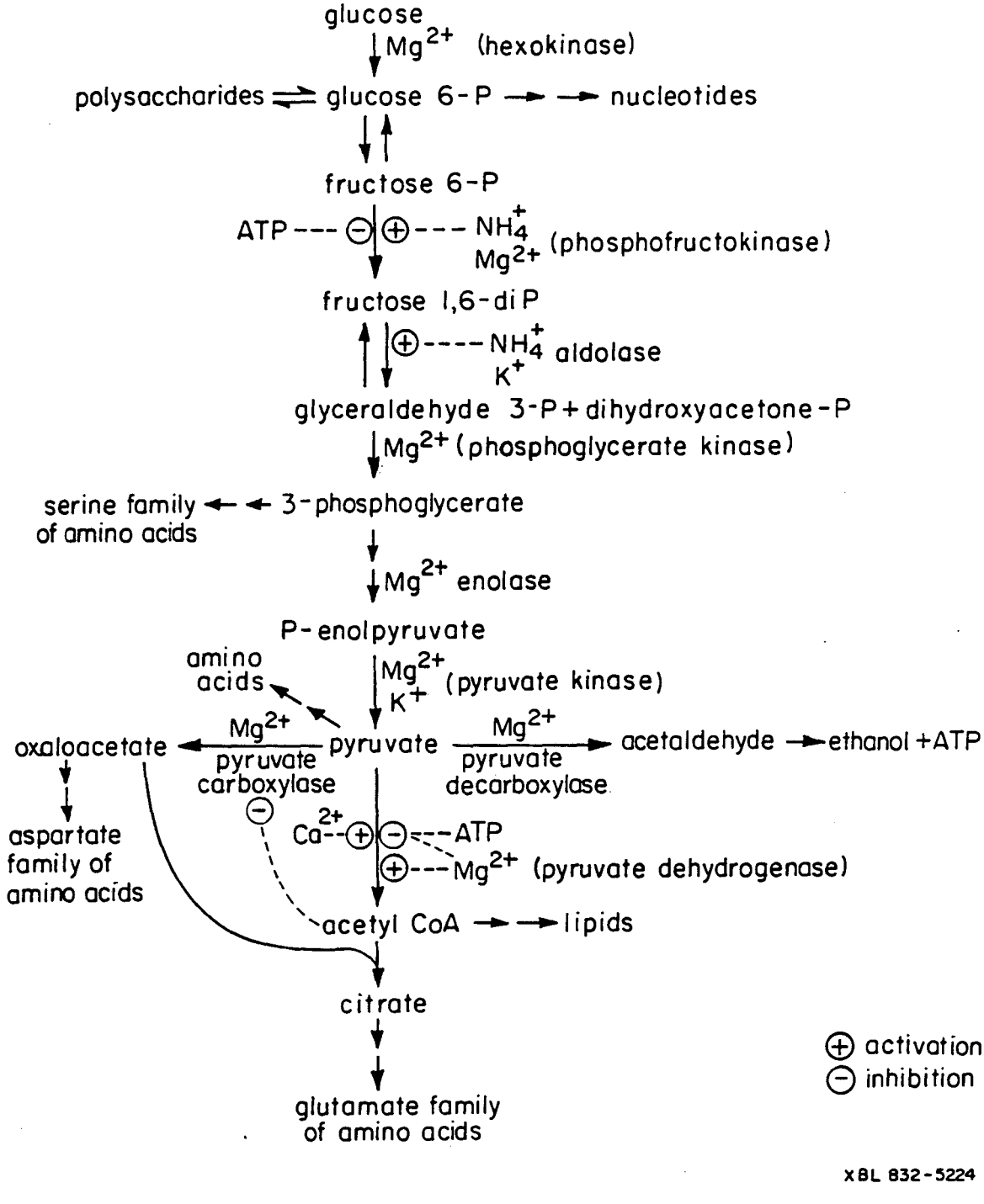

Figure 7.3 Inorganic Ions as Cofactors for Catalyzing Pathways to Production of Ethanol and Cell Mass Components

Table 7.7

Function of Required Growth Factors

Growth factor	Coenzymes or active form	Enzymes requiring coenzyme or active form	Function	Reference
Biotin	Biotin	Pyruvate carboxylase, Acetyl-CoA carboxylase	Carboxyl transfer	4, 18
Ca-pantothenate	Coenzyme A	Citrate synthetase, Pyruvate dehydrogenase, Fatty acid synthetase	Acyl group transfer	4,13,14,18
Pyridoxine	Pyridoxal phosphate	Transaminases	Amino group transfer	4,14,18
Thiamine	Thiamine pyrophosphate	Transketolase, Pyruvate dehydrogenase, Pyruvate decarboxylase	Aldehyde group transfer	4,14,18
Myo-inositol	Phosphatidyl inositol	Phosphofructokinase (?)	effector for lipid biosynthesis	20,21,22

Table 7.8

Functions of Major Inorganic Ions

Inorganic Ion	Cofactor or Effector for	Structural Component of	References
NH_4^+	phosphofructokinase (counteracts inhibition by ATP), aldolase (stimulates)	proteins, nucleic acids	18, 19
K^+	pyruvate kinase, aldolase (stimulates), membrane ATPase	ribonucleic acids	18, 19
PO_4^{-3}	ATP	nucleic acids, phospholipids, cell wall polymers	23
Mg^{+2}	hexokinase, phosphofructokinase, phosphoglycerate kinase, enolase, pyruvate decarboxylase, pyruvate kinase, membrane ATPase, pyruvate carboxylase	ribosomes, cell membranes, nucleic acids	18, 19, 24
Ca^{+2}	pyruvate dehydrogenase phosphatase, proteinase	cell wall proteins	13, 18, 25

components on various fermentation parameters.

Using the fermentor working volume as the control volume, the proposed model relates the specific cell mass production rate to the concentrations of cell mass components derived from a combination of parallel and series pathways. Although there are fewer pathways to ethanol, the specific ethanol production rate is similarly related to the concentration of its precursors. Furthermore, the concentrations of each intermediate in the pathway to cell components, ethanol or other by-products are functions of the concentrations of their precursors and of the available ATP if energy is required for their production.

For example, the specific rate of cell mass production (μ) is related to the specific rates of production of its protein, lipid, nucleic acid and polysaccharide components with respect to total cell mass (μ_i's). In turn, the μ_i's for protein, lipid, nucleic acid and polysaccharide are related to the concentrations of amino acids, fatty acids, nucleic acids and monosaccharides, respectively, and the concentration of available ATP. Eventually these precursors can be traced back to points in the pathways where the feed components enter either as substrates or catalytic factors.

Furthermore, the specific ethanol production rate is linked to the specific energy consumption rate for cell growth and maintenance. The energy for maintenance normally should be much less than that for growth. Based on the maintenance coefficients reported by Solomon and Erickson (26) and by Pirt (23) for Saccharomyces cerevisiae growing on glucose under fermentative conditions, the maintenance energy requirement should be less than 2% of the total energy consumed as glucose in

this work. However, Watson (27) and Maiorella et al. (28) showed that adverse conditions, such as, by-product or substrate inhibition could increase the maintenance energy considerably.

The specific rate of energy consumption for cell growth is determined by the pathway, which is dependent on the feed medium composition. Substrates in the form of pre-assembled cell components should utilize simpler pathways and less energy. Deficiencies in required growth factors may force utilizing alternate pathways leading to different products and/or different energy requirements. Each set of pathways to cell mass production can have a different efficiency, which was defined by Maiorella et al. (28) as

E = cell mass produced/ ethanol produced

and thought of as "the overall efficiency with which the cell utilizes its available energy for new cell mass production." This efficiency is the reciprocal of the yield coefficient $Y_{p/x}$ relating product to cell mass production.

The mechanism which links the specific ethanol production rate to the specific growth rate is illustrated in Figure 7.2. Cascade feed back inhibition of phosphofructokinase and of the glucose carrier into the cell regulates the ATP supply. Buildup of ATP inhibits glucose uptake.

The ethanol yield $Y_{p/s}$ can be interpreted as the ratio of the specific ethanol productivity to the specific glucose consumption rate. Similarly, the cell mass yield $Y_{x/s}$ is the ratio of the specific growth rate to the specific glucose consumption rate. The specific glucose consumption rate is the sum of the specific rates for conversion to cell

mass, ethanol and other by-products. The specific ethanol productivity and the specific growth rates are related to specific glucose consumption rates for ethanol and growth, respectively, through the stoichiometry of glucose conversion to ethanol and cells. Cells are assumed 50% carbon by dry weight.

To illustrate the conceptual metabolic model in general mathematical terms, the following representation may be postulated. All concentrations and specific rates are with respect to the fermentor working volume.

Specific cell mass production rate:

$$\mu = \sum_i \mu_i$$

$$\mu_i = \frac{1}{X}\frac{dX_i}{dt} = \sum_j f_{ij}(S_{ij}, ATP)$$

where

μ = specific cell mass production rate

μ_i = specific production rate of cell mass component i with respect to total cell mass

X = concentration of total cell mass

X_i = concentration of cell mass component i

t = time

f_{ij} = function relating μ_i to S_{ij} and ATP

S_{ij} = concentration of precursor j of cell mass component i

ATP = concentration of available ATP

Specific ethanol production rate:

$$q_p = \sum_p f_p(S_p, ATP)$$

$$q_p = Y_{p/x}\mu + Y_{p/s}\, m$$

where

q_p = specific ethanol production rate

f_p = function relating q_p to S_p and ATP

S_p = concentration of precursor p of ethanol

$Y_{p/x}$ = ethanol yield with respect to cell mass

$Y_{p/s}$ = ethanol yield with respect to glucose

m = maintenance coefficient

Specific glucose consumption rate:

$$q_s = q_{s,p} + q_{s,x} + q_{s,b}$$

where

q_s = total specific glucose consumption rate

$q_{s,p}$ = specific glucose consumption rate for ethanol production

$q_{s,x}$ = specific glucose consumption rate for cell mass production

$q_{s,b}$ = specific glucose consumption rate for by-product production

Ethanol and cell mass yields:

$$Y_{p/s} = q_p / q_s = 1.96\, q_{s,p} / q_s$$

$$Y_{x/s} = \mu / q_s = 1.25\, q_{s,x} / q_s$$

where

$Y_{x/s}$ = cell mass yield with respect to glucose

In the above model the specific production rate of each cell component is a sum of functions of intermediate precursors and ATP concentrations. Each function in the sum represents a parallel path to the cell component. Each of the parallel paths in turn can be considered a subcombination of series and parallel reaction steps. If the subcombination for a main parallel path can be considered as a series reaction,

the corresponding function f_{ij} (S_{ij}, ATP) can be represented as a product function. For illustration purposes, if each reaction in the series shows saturation kinetics with respect to its limiting substrate, f_{ij} can be expressed as a product of Monod type expressions and a function of the energy available as ATP, e(ATP), as follow:

$$f_{ij}\ (S_{ij},\ ATP) = e(ATP)\ \prod_j \mu_{imax}\ S_{ij} / (S_{ij} + K_{ij})$$

where

μ_{imax} = maximum specific rate of production of cell mass component i

K_{ij} = saturation constant for precursor j of cell mass component i

The exact kinetics of this example, as determined by the reaction rate constants, depend on the concentration of the enzymes, cofactors and effectors.

Neither the pathways depicted in Figures 7.2 and 7.3 nor the equations in the above mathematical model are meant to be complete descriptions of the kinetic mechanisms of cell growth or ethanol production. They are attempts to provide a framework for understanding the qualitative effects of the major classes of nutrients studied.

7.4.2 Results and Discussion

The effects of the feed concentration of individual nutrients on specific ethanol productivity, cell mass and ethanol yields, cell mass and ethanol concentrations, and residual glucose concentrations or fraction glucose utilized are summarized in Figures 7.4 to 7.15. To understand the effect of each nutrient on the above fermentation parameters, it is helpful to classify the nutrients into the roles specified in Table 7.9, which also lists the nutrients, their types, and the feed

Table 7.9

Classification and Concentration Ranges of Nutrients Studied

Medium Component	Active Nutrient	Nutrient Type	Nutrient Role	Range of Feed Concentration for Medium Component
Biotin	Biotin	Growth factor	Cofactor	2.72 - 56.2 μg/L
Ca-pantothenate	Ca-pantothenate	Growth factor	Cofactor	0.063 - 6.92 mg/L
Thiamine	Thiamine	Growth factor	Cofactor	0 - 8.00 mg/L
Pyridoxine	Pyridoxine	Growth factor	Cofactor	0 - 6.66 mg/L
Myo-inositol	Myo-inositol	Growth factor	Cofactor, substrate	0.125 - 20.0 mg/L
$(NH_4)_2SO_4$	NH_4^+	Inorganic ion	Substrate, effector	0.885 - 6.20 g/L
KCl	K^+	Inorganic ion	Substrate, cofactor	0.0368 - 0.625 g/L
H_3PO_4	PO_4^{-3}	Inorganic ion	Substrate, cofactor	0.411 - 0.738 g/L
$MgSO_4 \cdot 7H_2O$	Mg^{+2}	Inorganic ion	Substrate, cofactor	0.124 - 0.331 g/L
$CaCl_2 \cdot 2H_2O$	Ca^{+2}	Inorganic ion	Substrate, cofactor	0.0295 - 0.060 g/L
Yeast Extract	Many	Complex components	Precursors, substrates, cofactors	1.0 - 19.4 g/L
Cornsteep Liquor	Many	Complex components	Precursors, substrates, cofactors	0 - 71.0 g/L

concentration ranges studied.

a) Cofactors and Effectors

Cofactors and effectors are characterized by their effects in low concentrations on the catalytic activity of enzymes. Either as metal ions or organic coenzymes, cofactors are commonly required for enzyme activity. Furthermore, Witt and Neufang (29) reported that thiamine phosphates could induce synthesis of the protein moeity of pyruvate decarboxylase in Saccharomyces cerevisiae. They also cited Greengard and Gordon's report (30) that pyridoxine could induce the pyridoxal phosphate dependent tyrosine-α-ketoglutarate transaminase in rat liver. Effectors modulate the activity of allosteric enzymes through non-covalent binding; they can stimulate or inhibit activity.

Depending on the location of the enzymes in the pathways of the organism, these activity changes can affect the specific growth rate and/or the specific ethanol production rate. As seen in Figure 7.2, the only required vitamin along the only pathway to ethanol and away from cell mass components, i.e., from pyruvate to ethanol is thiamine. Therefore, thiamine could possibly stimulate the specific rate of ethanol production by diverting more pyruvate from cell mass components to ethanol. Because thiamine is also required for the pentose phosphate pathway and for the oxidative decarboxylation of pyruvate to acetyl CoA, thiamine could, but may not, increase the ethanol to cell mass ratio. However, all other required vitamins catalyze pathways to preferentially produce cell mass to ethanol.

Because the specific growth rate is fixed for constant dilution

rate in chemostat culture, the ratio of ethanol to cell mass produced is proportional to the specific ethanol productivity q_p. As seen in Figures 7.4 to 7.8, q_p increased from addition of thiamine and pyridoxine, but decreased from addition of biotin, Ca-pantothenate and myo-inositol. With the exception of the pyridoxine result, these results are consistent with the role of the required vitamins as proposed in the Figure 7.2.

The increased q_p from addition of pyridoxine may be explained by the interrelationships of pyridoxine and thiamine found by Moses (31) and Moses and Joslyn (32) in one strain of Saccharomyces cerevisiae. They found that consumption of pyridoxine by a culture was accompanied by synthesis of thiamine and vice versa. Thus, either growth factor could partly spare the requirement for the other to attain an appreciable growth rate. However, both pyridoxine and thiamine were required for the maximum growth rate. They postulated a reversible relationship between pyridoxine and thiamine as follows. Pyridoxine both reversibly converts to pyrimidine and stimulates the rate of synthesis of thiazoles. Then pyrimidine condenses with thiazole to form thiamine. Hence, pyridoxine is important both catalytically and stoichiometrically in thiamine biosynthesis. Suomalainen and Oura (38), Hough et al. (40), and Atkin (41) also reported that thiamine and pyridoxine were alternatives for certain strains of yeast.

The pyridoxine data in Figure 7.6 were obtained with 1.34 mg/L thiamine in the feed. According to the data in Figure 7.7 (obtained with 3.0 mg/L pyridoxine in the feed), this feed thiamine concentration was limiting to the specific ethanol productivity. Therefore, pyridoxine up

to 3.0 mg/L in the feed may have been required for producing additional thiamine for q_p up to the constant maximum level of 0.96 g-etoh/g-cell-hr. This was approximately the constant maximum q_p (0.93 g-etoh/g-cell-hr) for thiamine in Figure 7.7. Increasing the feed pyridoxine past 3.0 mg/L may have decreased q_p from its maximum point because of the role of pyridoxine in stimulating the specific cell growth rate.

For given feed substrates, the ethanol and cell mass yields with respect to glucose are determined by the relative effects of growth factors in catalyzing competing reactions for producing cell mass and ethanol from glucose. As shown in Figures 7.4 to 7.8, the cell mass yield was independent of the concentrations of thiamine and pyridoxine, but dependent on the concentrations of biotin, pantothenate and myo-inositol at low levels. Thus, the cell mass yields reached maximum constant levels for sufficient concentrations of all required growth factors. At lower concentrations of the growth sensitive growth factors, i.e., biotin, Ca-pantothenate, and myo-inositol, the cell mass yield declined. This decreased cell mass yield could be explained for Ca-pantothenate and myo-inositol in terms of reduced rates of reactions for which they are cofactors and which lead to cell mass components. In the case of biotin, however, the lower end of the feed concentration range studied was less than the saturation constant K_m reported for biotin uptake in Table 7.6. Hence, there was a mass transfer limiting effect for biotin as well as a possible reaction rate limiting effect which could have affected cell mass yield. Maximum constant cell mass yield for increased growth factor concentrations could be explained by new limitations in other reaction factors, such as the apoenzyme, for reaction rate controlled growth and elimination of all mass transfer

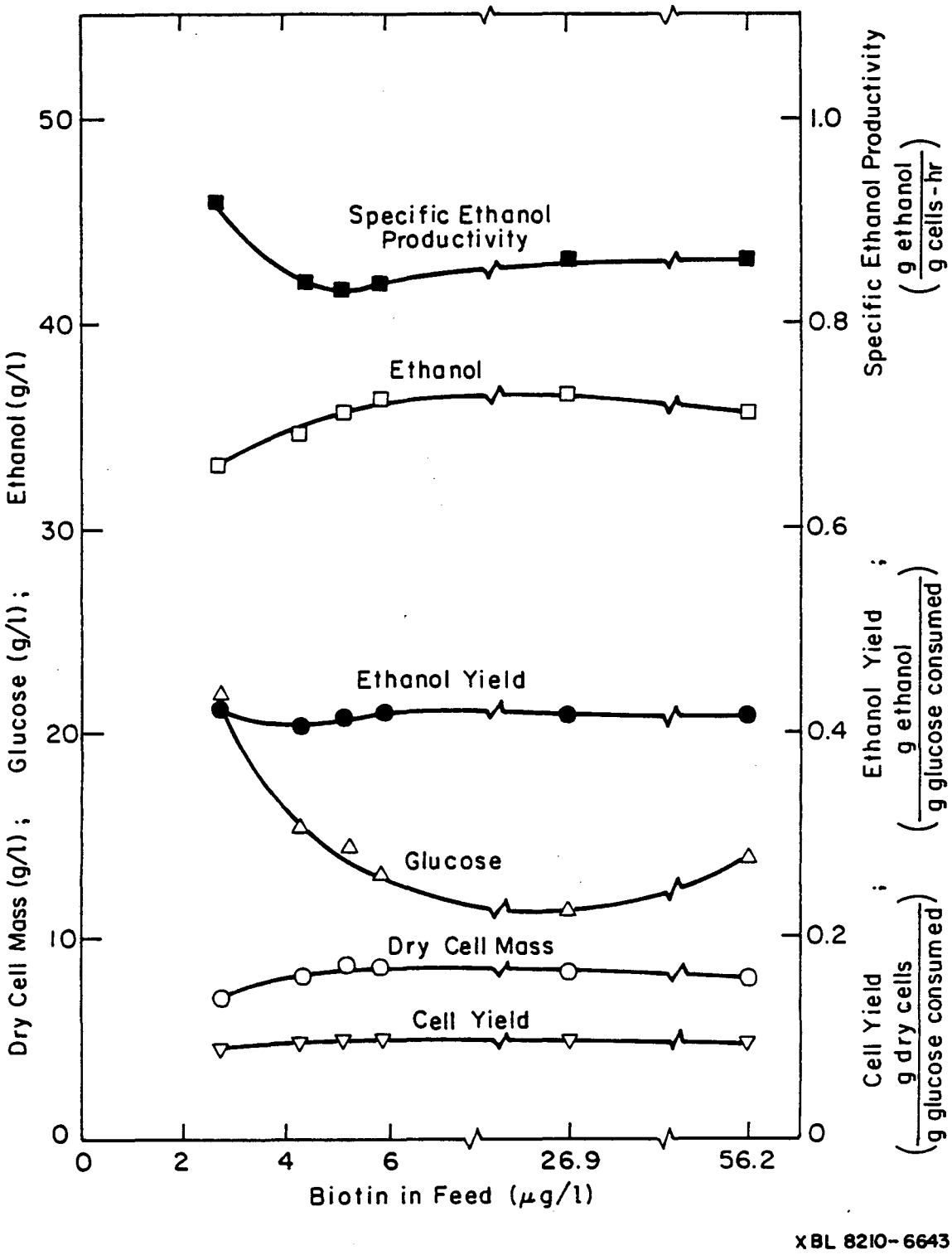

Figure 7.4 Effect of Biotin Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

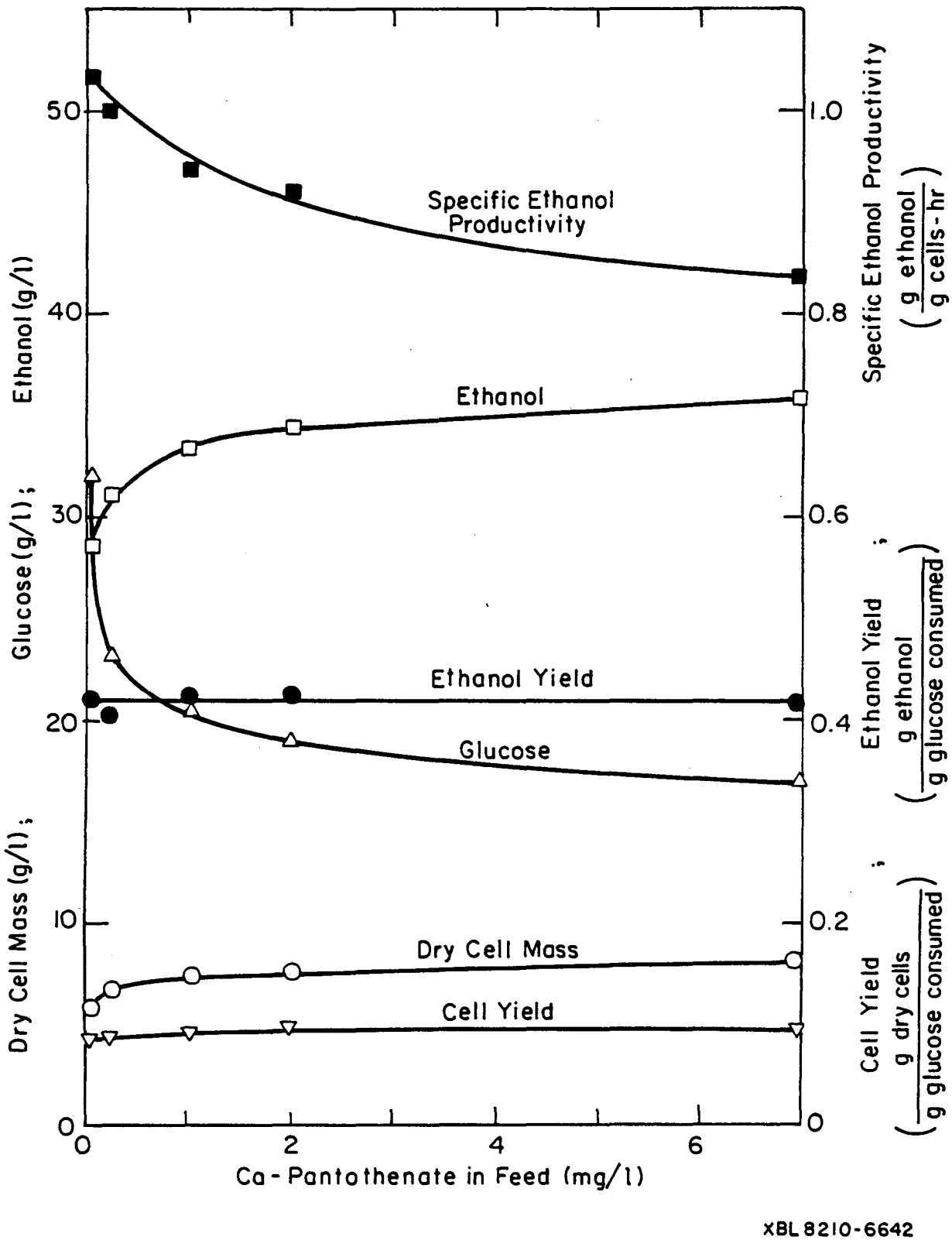

Figure 7.5 Effect of Ca-Pantothenate Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

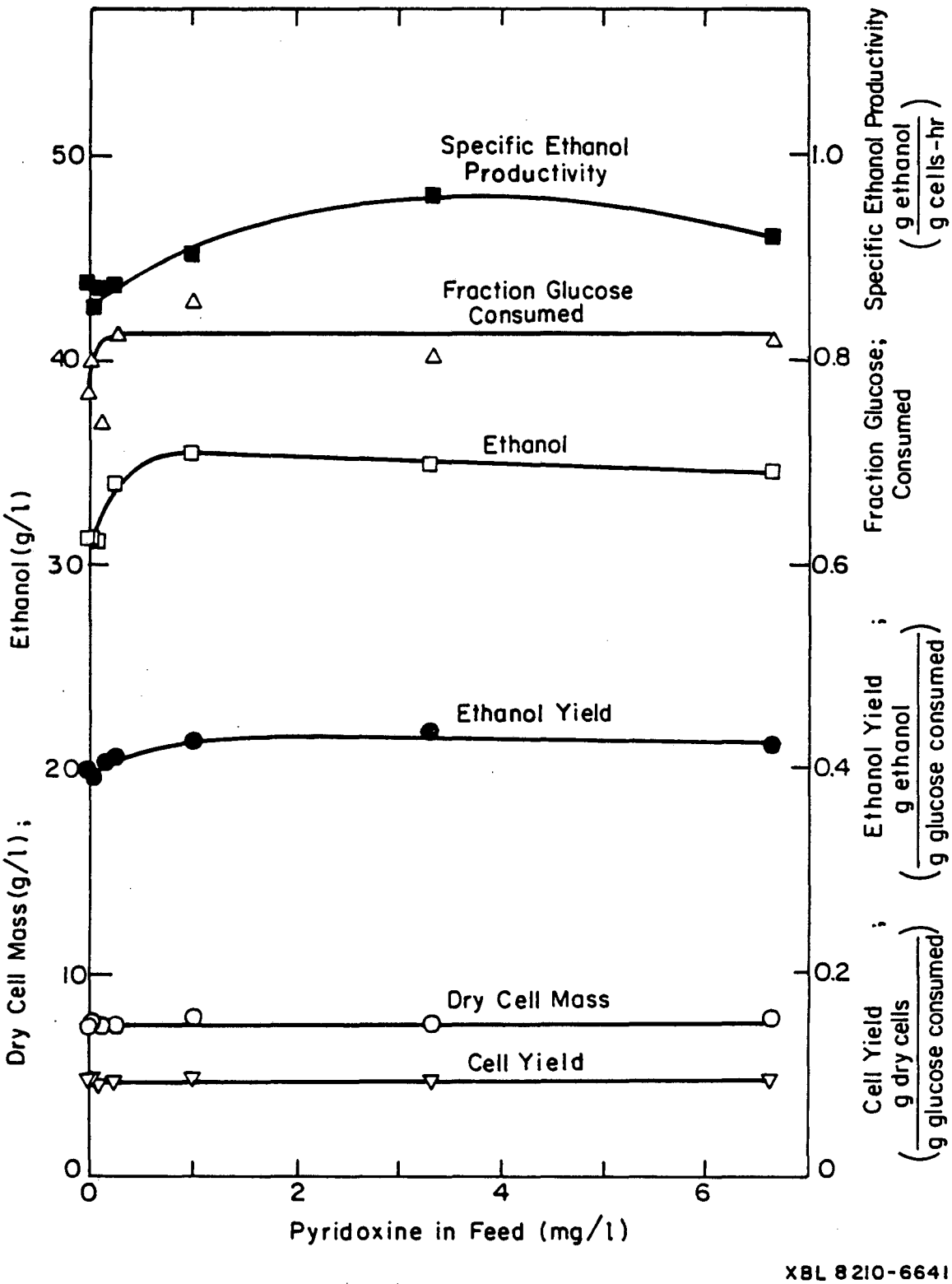

Figure 7.6 Effect of Pyridoxine Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

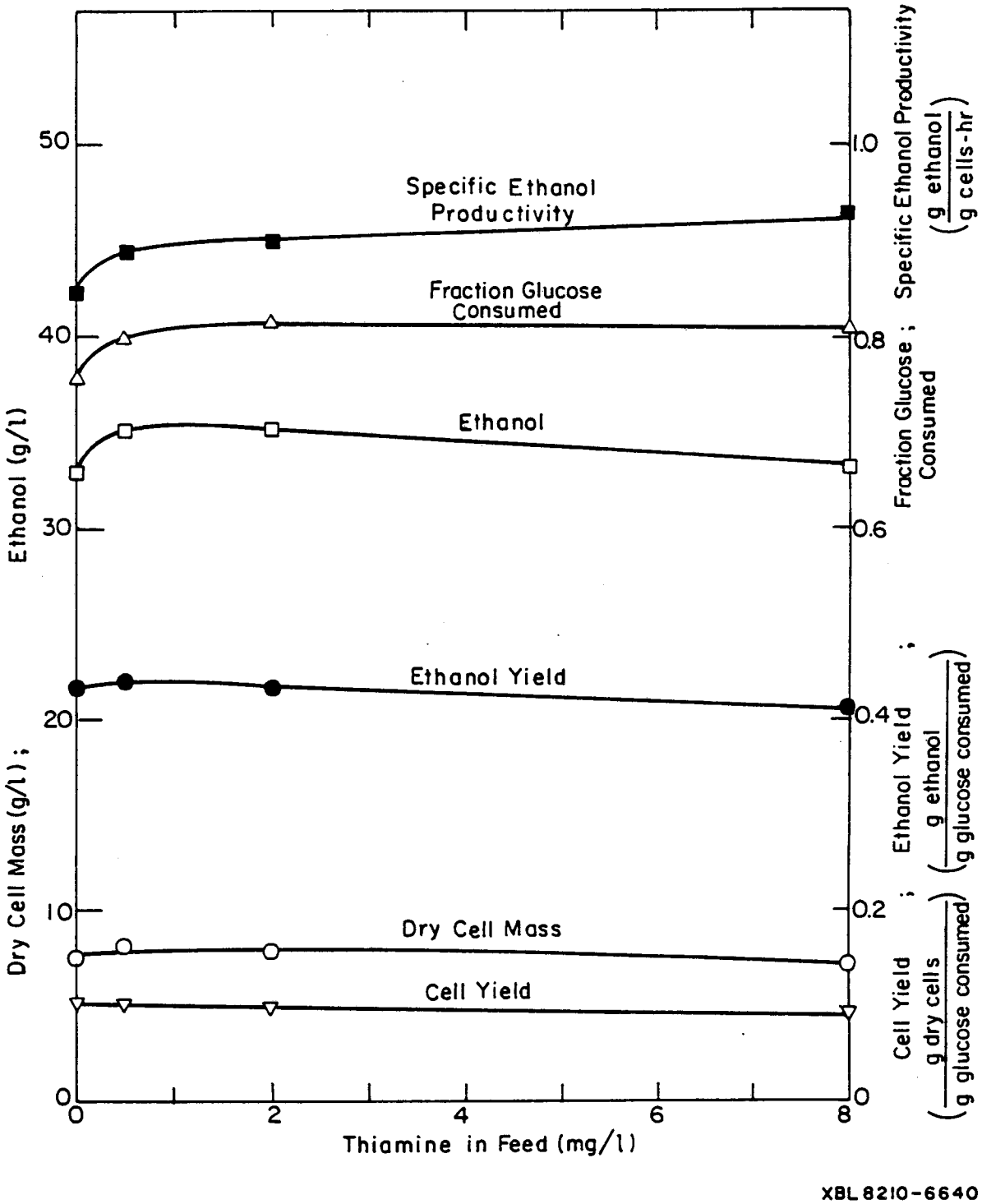

Figure 7.7 Effect of Thiamine Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

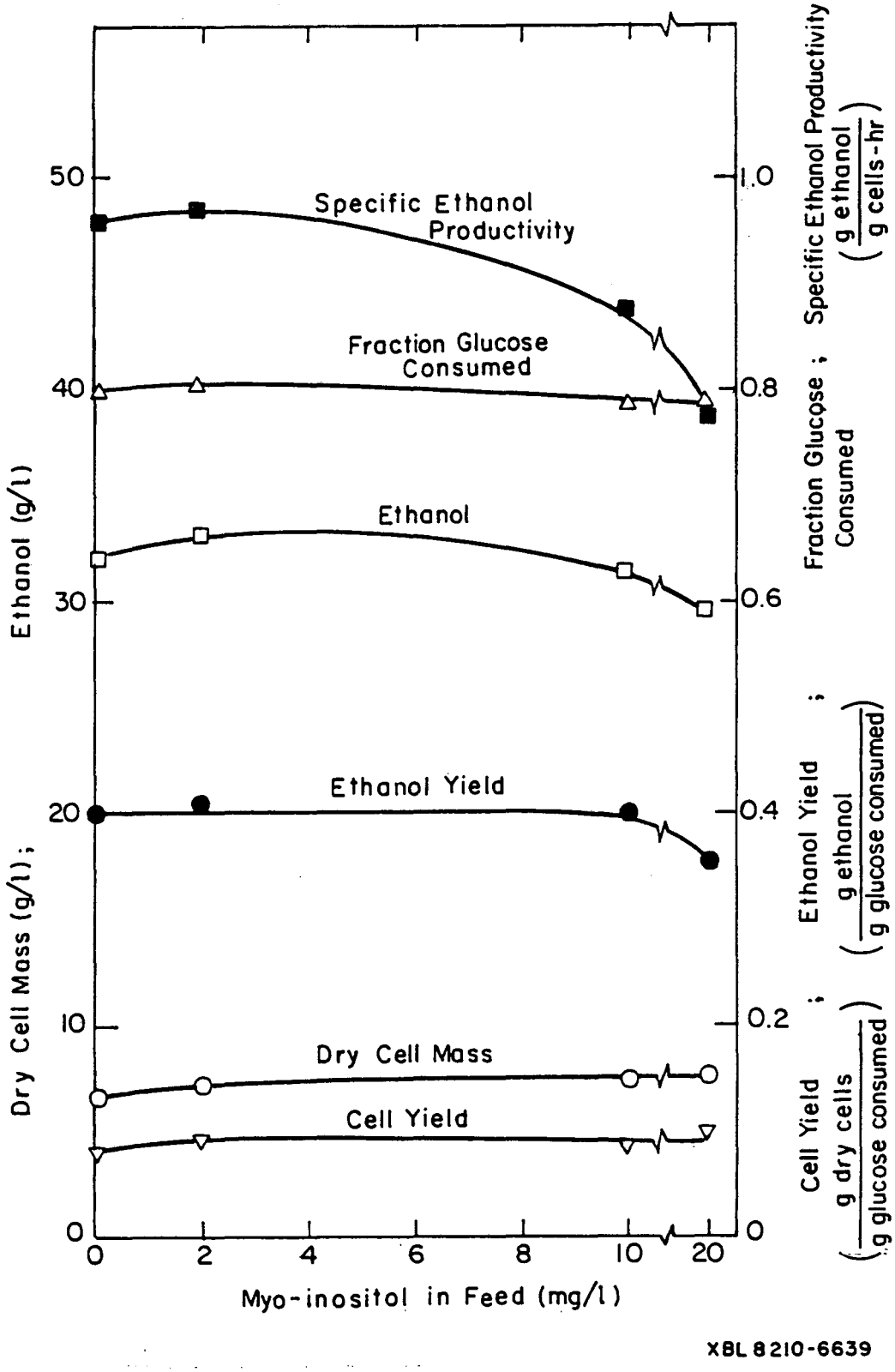

Figure 7.8 Effect of Myo-inositol Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

limitations.

The ethanol yield appeared almost independent of the concentrations of each of the growth factors individually with two exceptions, namely, low concentrations of pyridoxine and high concentrations of myo-inositol. For less than 1 mg/L pyridoxine and 1.34 mg/L thiamine in the feed, the ethanol yield showed a slight decline from the maximum constant level. This limitation was consistent with the preferential stimulation of the specific ethanol production rate to the specific growth rate noted above for pyridoxine and thiamine. Furthermore, as with biotin, both transport of pyridoxine into the cell at low feed concentrations and catalysis of reactions within the cell may have been limiting, in this case, to the ethanol production rate. At the highest concentration studied (20 mg/L), myo-inositol may have changed from a catalytic role to a substrate for cell mass since it reportedly has both functions. Relative stimulation of cell mass to ethanol production by the substrate could explain the increase in cell yield and the decrease in ethanol yield.

b) Substrates

Substrates are simple medium nutrients (relative to precursors) which are transformed by enzymes to intermediates of cell components or by-products. The special class of substrates considered in this study is the inorganic ion required as a macronutrient. They are listed with their functions in Table 7.8. All these ions serve both as cofactors and effectors for enzymes and as major substrates for structural components of the cell. These two functions must be separated to determine the substrate role of inorganic ions.

The critical role of these ions as catalytic cofactors in major pathways to ethanol and cell mass components are summarized in Figure 7.3. They are essential to the activity of many of the glycolytic enzymes in converting glucose to pyruvate. They are also required for converting pyruvate to acetaldehyde, which is the precursor to ethanol, or to oxaloacetate and acetyl CoA, which are precursors to cell components. Inorganic ions in excess as cofactors or effectors are assumed to have little effect on specific rates or yield as demonstrated above for cofactors and effectors.

Since the inorganic ions were used also as major substrates requiring high feed concentrations, there should not have been any deficiencies of these ions as cofactors or effectors. Therefore, the effects of varying feed concentrations of the major inorganic ions shown in Figures 7.9 to 7.13, were mainly derived from the substrate functions of the ions.

As seen in Figures 7.9 to 7.11, the specific ethanol productivity declined with increasing concentrations of ammonium, potassium and phosphate ions just as it declined with increasing concentrations of the growth sensitive cofactors. By far, these are the ions in highest concentration in yeast according to the analysis given by Harrison (33). Thus, increased concentrations of these ions could increase rates for reactions leading to cell mass relative to those leading to ethanol.

Increased potassium ion concentration had an especially great effect on cell growth rate, cell mass yield and specific ethanol productivity. In addition to the stimulatory effect on cell growth promoting reactions, the transport rate of potassium into the cell should have

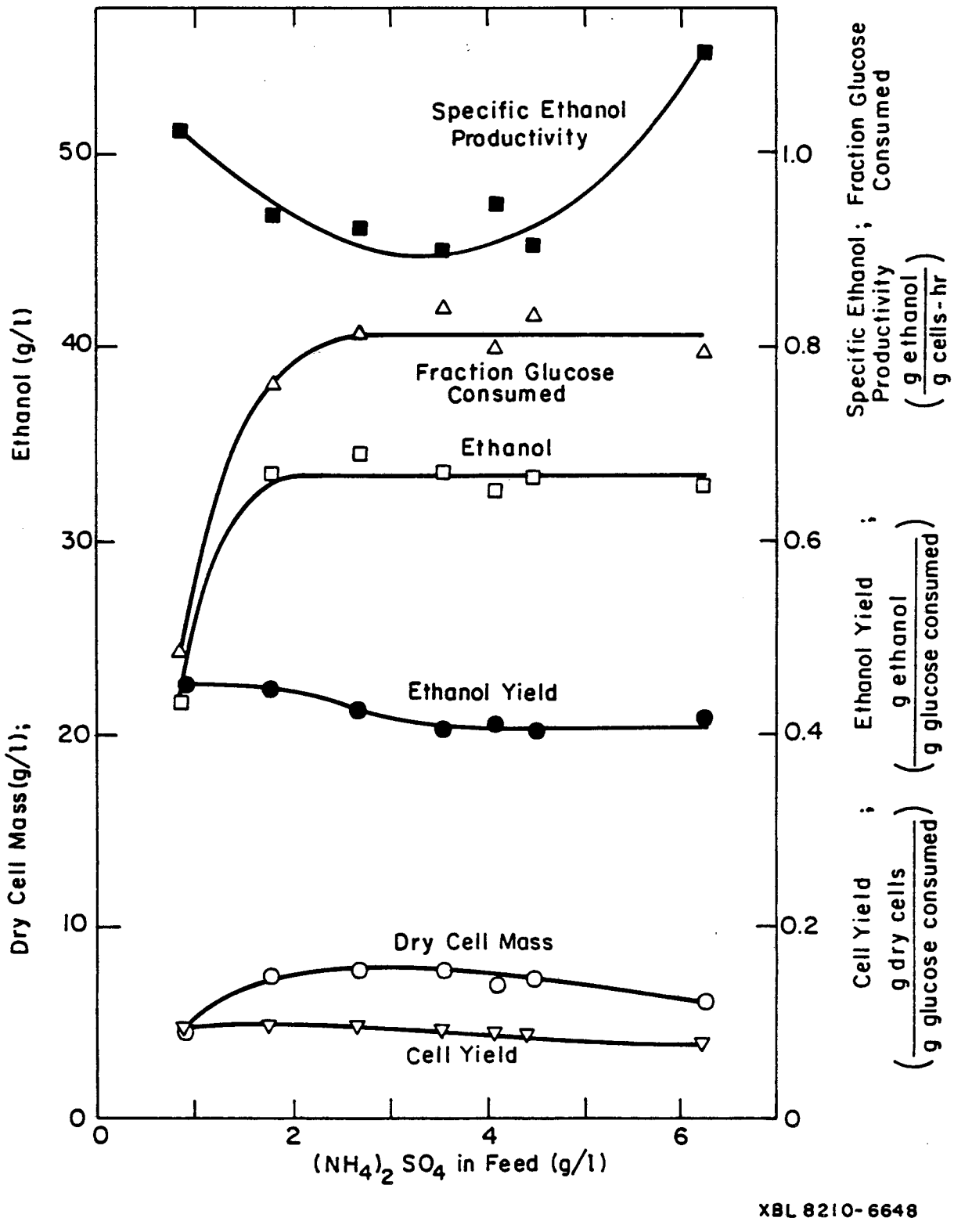

Figure 7.9 Effect of Ammonium Sulfate Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

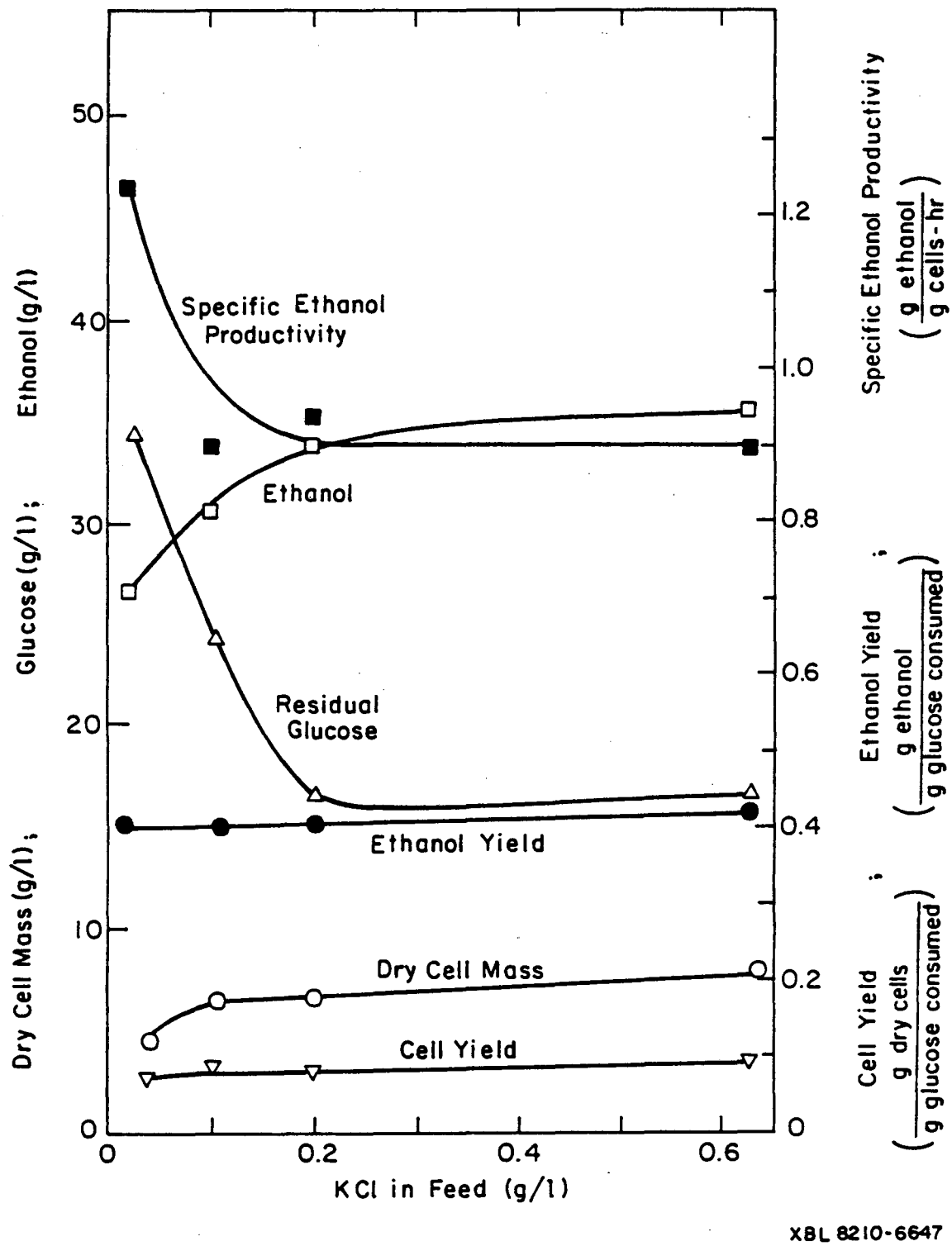

Figure 7.10 Effect of Potassium Chloride Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

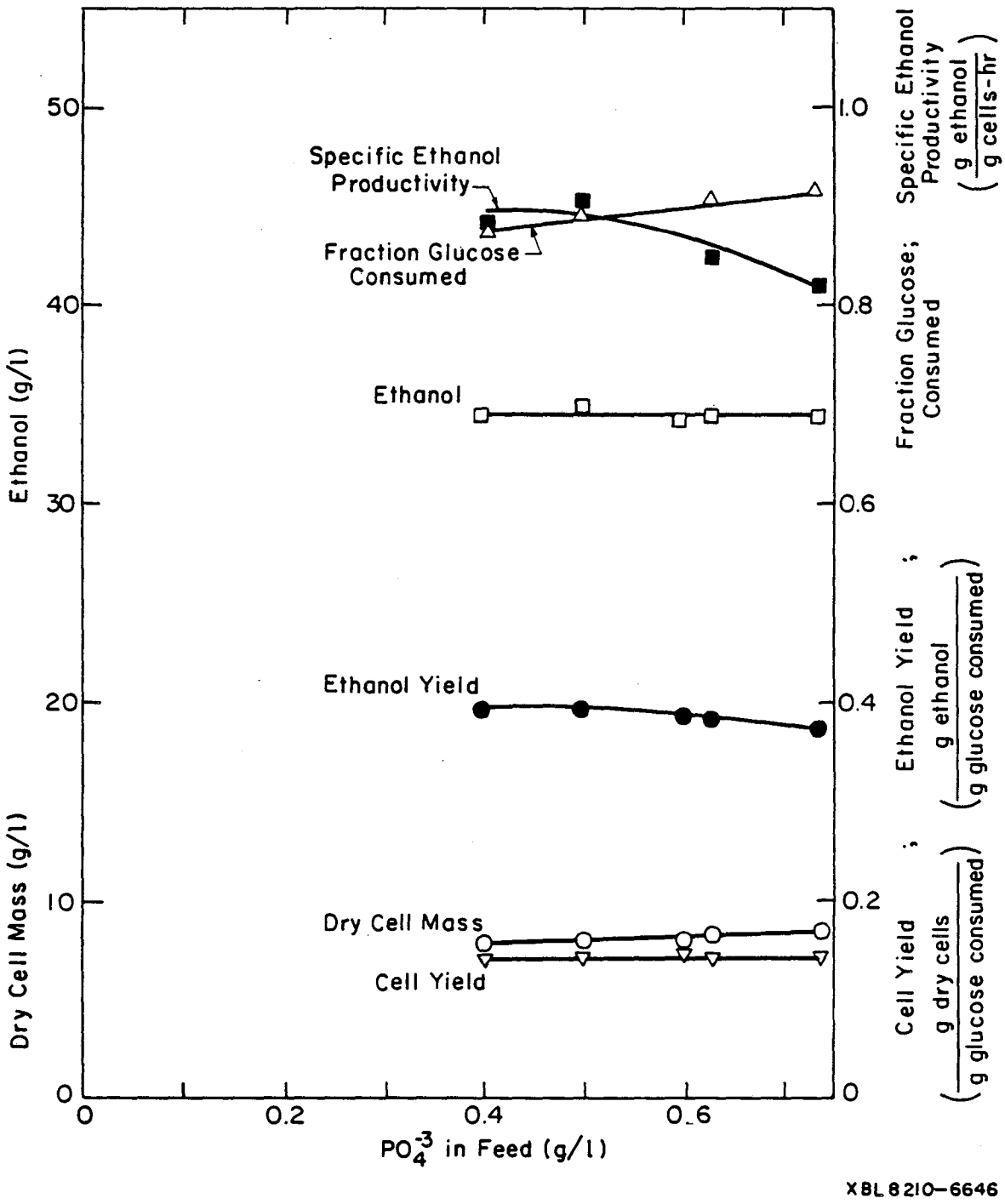

Figure 7.11 Effect of Phosphate Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

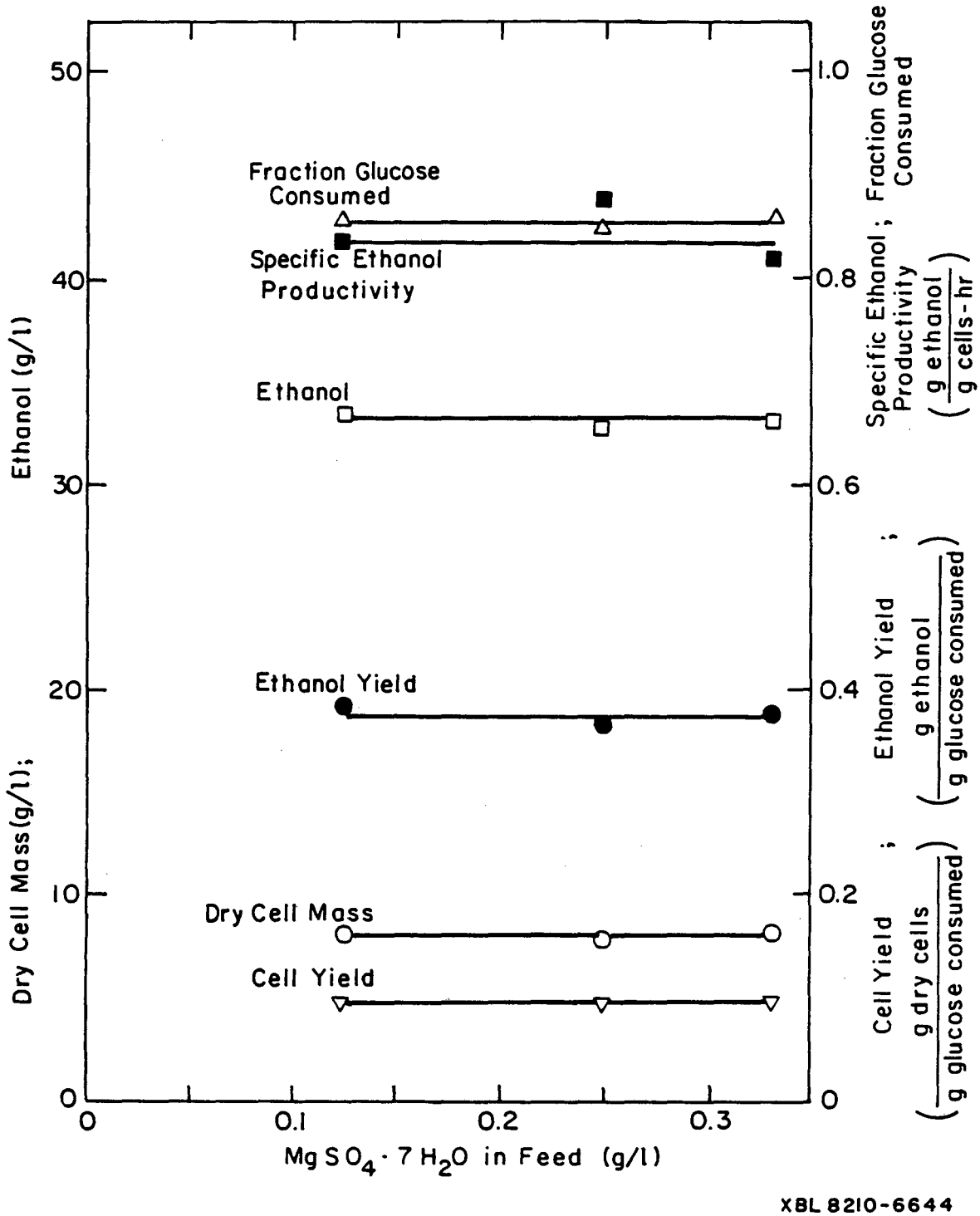

Figure 7.12 Effect of Magnesium Sulfate Heptahydrate Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

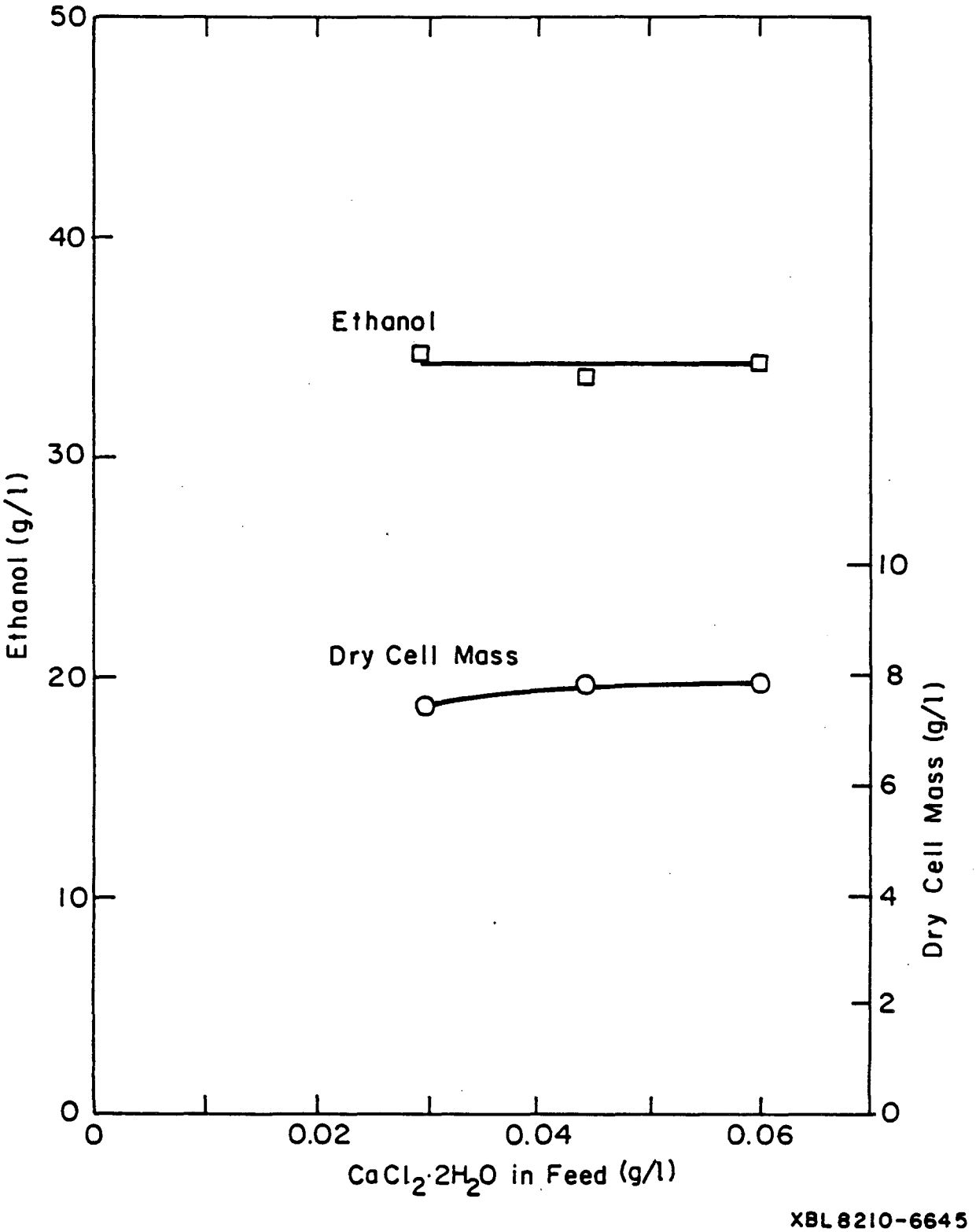

Figure 7.13 Effect of Calcium Chloride Dihydrate Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

increased considerably. The saturation constant K_m for potassium (0.2 - 0.4 g/L) given in Table 7.6 is greater than or of the same order as the feed concentration range studied (0.019 - 0.325 g/L as K^+). Thus mass transfer limitation effects are significant in this range. Feed concentrations of all the other ions are much greater than their K_m's.

Furthermore, the declining specific ethanol productivity with increased ion concentrations may have been due to increased energy requirements for transporting low concentrations of ions against high concentration gradients. A mechanism involving membrane bound ATPase to provide energy for transport of various ions has been proposed by Pena (34) and Kotyk and Horak (35). Wumpelmann and Kjaergaard (36) reported an even steeper decline in specific ethanol productivity by increasing the potassium feed concentration from 2.5 to 9.5 mM. They found the potassium content of the yeast unaffected by the concentration change and suggested that the concentration gradient across the cell membrane strongly affected fermentation activity.

The cell mass concentration, which is directly proportional to the cell mass productivity at constant dilution rate, and the cell mass yield reached maximum constant levels for sufficient feed inorganic ion concentrations with the exception of ammonium sulfate. The saturation effect for cell mass production reflected non-limiting substrate concentrations with respect to cell mass producing reactions for all ions except ammonium and with respect to transport of substrate for potassium ions.

The ethanol yields were either constant or slightly declining (ammonium sulfate and phosphate) with increased ion feed concentrations.

Ethanol yields are not expected to be significantly affected by these concentrations because the pathway for ethanol production does not require inorganic ions in substrate capacities. This pathway also is not in competition with pathways stimulated by substrate levels of ions for cell mass production. The declines in ethanol yield may have reflected the decreased energy requirement for transport of ions into the cell against lower concentration gradients.

The results for the ammonium sulfate study in Figure 7.9 are unique in showing inhibition effects. At the higher feed concentrations of ammonium sulfate studied, the ethanol and cell mass yields declined. These declines were consistent with the ammonium inhibition effects observed by Maiorella (37) over the feed concentration range equivalent to 0.78 to 16 g/L NH_4Cl for cell mass yield and 0.78 to about 11 g/L NH_4Cl for ethanol yield. The mechanism of ammonium inhibition is not clear, but ammonium ions are known to competitively counteract ATP inhibition of phosphofructokinase (16) and thus affect the regulation of glycolysis. Hence, the energy linkage connecting ethanol and cell mass production is disrupted.

c) Complex Components (precursors, cofactors, substrates)

Complex components are not chemically well defined mixtures of cofactors, substrates, precursors and unknown factors derived from organic sources. The two sources of complex components studied were yeast extract and cornsteep liquor; their effects are summarized in Figures 7.14 and 7.15.

The yeast extract study reflected mainly the effects of the

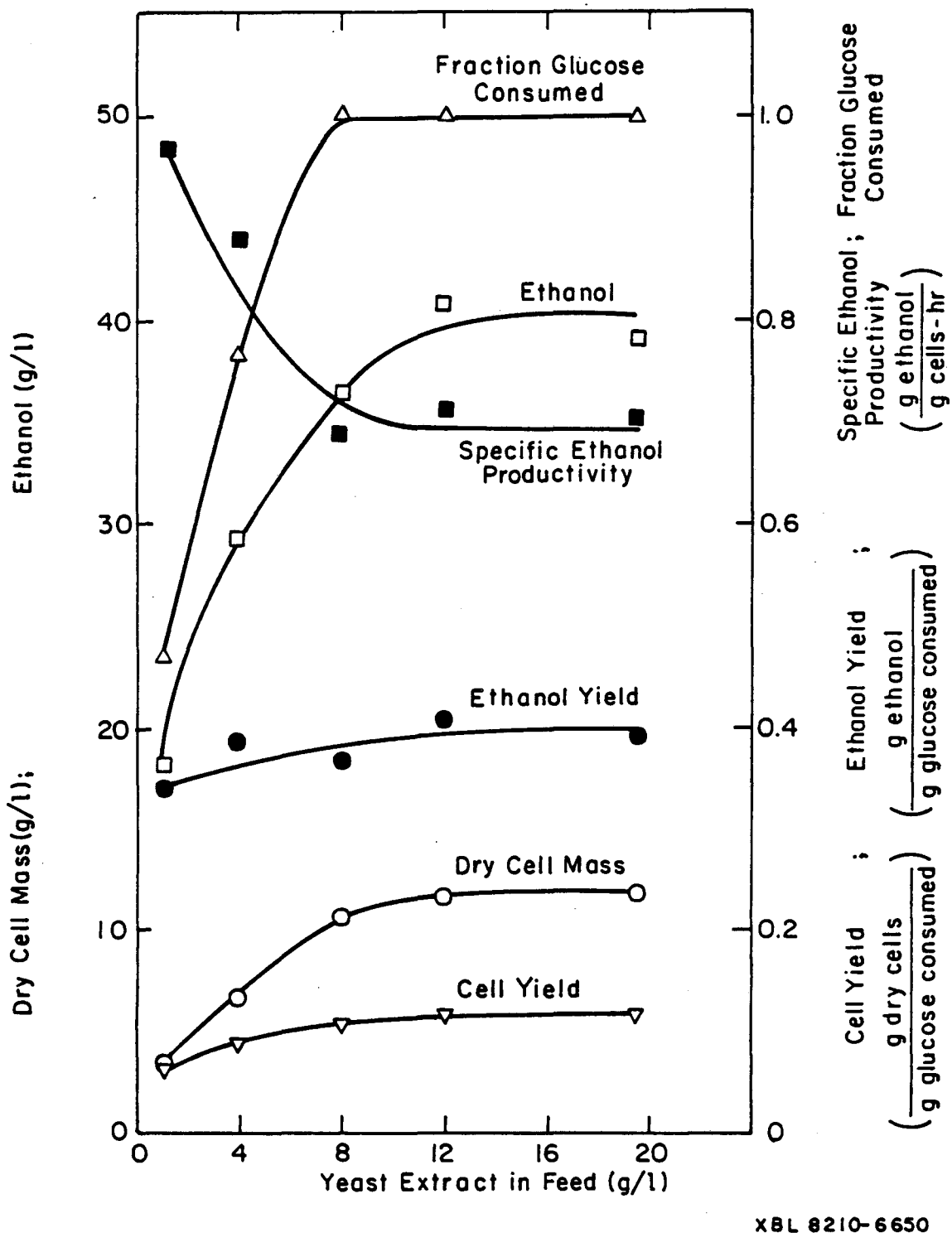

Figure 7.14 Effect of Yeast Extract Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

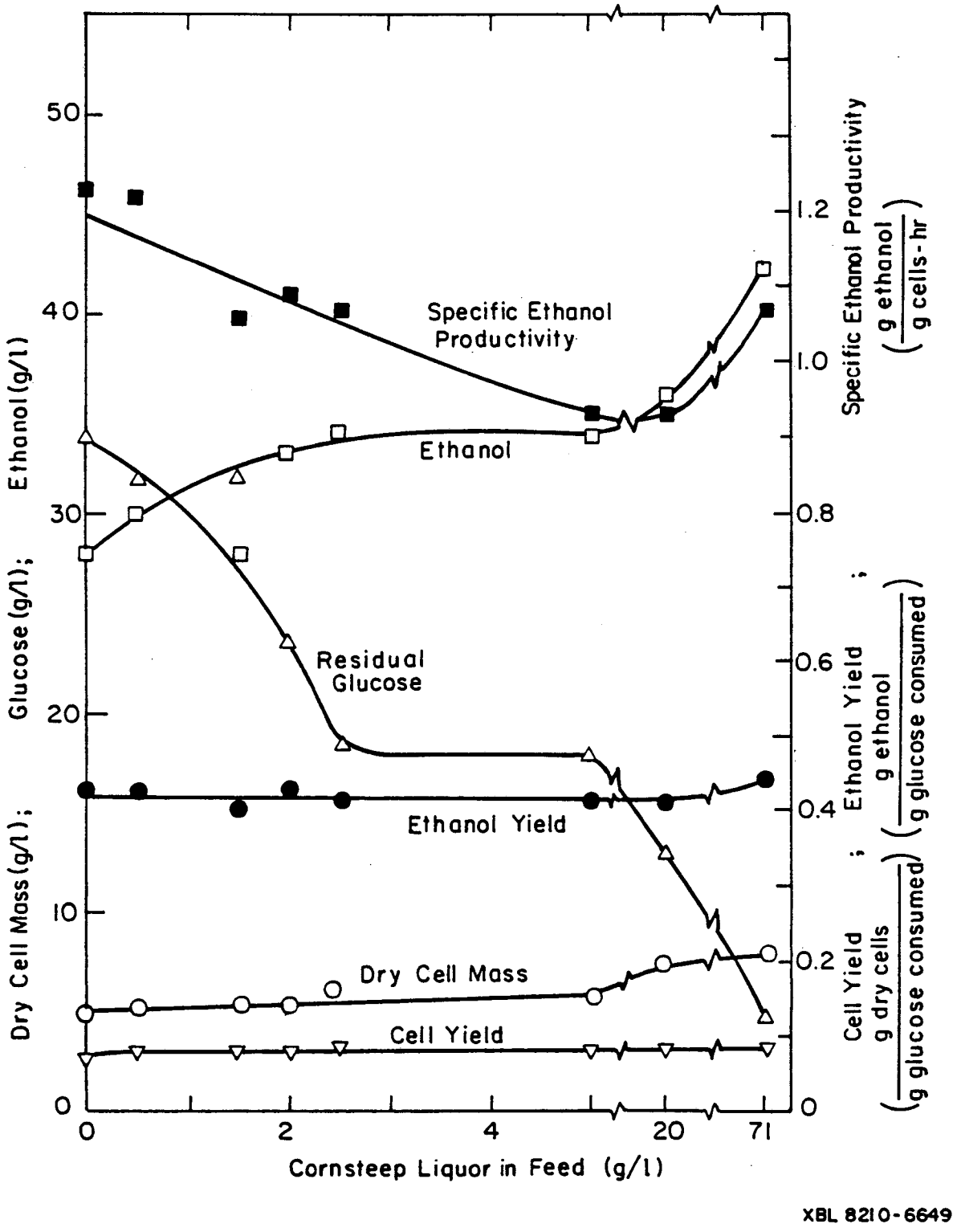

Figure 7.15 Effect of Cornsteep Liquor Feed Concentration in Continuous Culture of Saccharomyces cerevisiae

precursors, two required growth factors and unknown factors. The base medium for the yeast extract study contained sufficient minerals and required growth factors except thiamine and myo-inositol to be non-limiting. All known required growth factors were in the cornsteep liquor base medium, but the concentrations of certain inorganic ions were lower in a generally better balanced medium. Thus, the cornsteep liquor effects were mainly due to precursors and unknown factors.

Precursors consist of amino acids, nucleic acids and other readily assimilated building components of cell mass. By sparing glucose from cell mass production, precursors should increase both cell mass and ethanol yields with respect to glucose. This effect was observed to be less for cornsteep liquor than for yeast extract for three reasons. First, yeast extract is derived from yeast and should have more assimilable precursors. Second, the low cell mass and ethanol yields for low yeast extract feed concentrations may have resulted in part from myo-inositol and thiamine deficiencies. Third, the yeast used for the cornsteep liquor study was adapted to relatively synthetic media with less than 0.1 % complex components. This adapted yeast is less dependent on required growth factors and precursors. Thus, even at zero concentration cornsteep liquor, the ethanol and cell mass yields were close to the maximum yields attained at the highest concentration of cornsteep liquor used.

The decreased specific ethanol productivities for increases in both yeast extract and cornsteep liquor concentrations reflected the increased efficiency or the decreased energy requirements for growth from precursors. At 71 g/L cornsteep liquor in the feed, however, the

osmotic pressure effect may have increased the cell maintenance requirement and thus the specific ethanol productivity.

d) Combined Nutrients

From the data in Figures 7.4 to 7.15, the optimum feed concentration of each nutrient can be selected with respect to maximum specific ethanol productivity, ethanol yield and cell mass yield in Table 7.10 or maximum ethanol production, cell mass production and glucose consumption rates in Table 7.11. The media are optimum only with respect to the nutrients studied, but do not include all nutrients, such as trace elements required in synthetic media. For the nutrients studied, the concentrations given are optimum only with respect to the concentration range studied (Table 7.9). For equivalent performance, the lowest concentration is presented, but performance is not traded-off against cost. Because of interaction effects, combining all the individually optimum nutrient concentrations does not necessarily produce the overall optimum medium for a given fermentation parameter. The actual performance of the media in Tables 7.10 and 7.11 must be determined experimentally.

7.4.3 Conclusions

The effects of growth factor concentration on the fermentation parameters in Figures 7.4 to 7.8 were attenuated by the use of an adapted yeast. This yeast has developed enhanced capabilities for biosynthesis of growth factors previously required or stimulatory to cell growth or ethanol production. A completely synthetic medium with growth factors serving as stimulants in reduced concentrations can be utilized by this yeast at 0.20/hr dilution rate with resulting q_p = 1.2

Table 7.10

Summary of Media (100 g/L Glucose) for Maximum

Specific Ethanol Productivity, Ethanol Yield and Cell Yield

Nutrient	Conc. units	Feed conc.	Max q_p/hr	Feed conc.	Max $Y_{p/s}$	Feed conc.	Max $Y_{x/s}$
Biotin	µg/L	2.72	0.92	2.72	0.42	5.8	0.098
Ca-pantothenate	mg/L	0.063	1.0	0.063	0.42	6.92	0.097
Thiamine	mg/L	8.0	0.93	0.5	0.44	0.0	0.10
Pyridoxine	mg/L	3.33	0.96	3.33	0.44	0.125	0.096
Myo-inositol	mg/L	0.125	0.96	2.0	0.41	20.0	0.097
$(NH_4)_2SO_4$	g/L	0.885	1.0	0.885	0.45	1.77	0.098
KCl	g/L	0.0368	1.2	0.625	0.42	0.625	0.095
H_3PO_4	g/L	0.411	0.96	0.411	0.40	0.627	0.091
$MgSO_4 \cdot 7H_2O$	g/L	0.124	0.84	0.124	0.39	0.124	0.096
Yeast Extract	g/L	1.0	0.97	12.0	0.41	12.0	0.12
Cornsteep Liquor	g/L	0.0	1.2	71.0	0.44	3.0	0.086

Table 7.11

Summary of Feed Media (100 g/L Glucose) for Maximum Rates of Ethanol Production, Cell Mass Production and Glucose Consumption

Nutrient	Conc. Units	Feed Conc.	Max Etoh Production g/L-hr	Feed Conc.	Max Cell Mass Production g/L-hr	Feed Conc.	Max Glucose Consumption g/L-hr
Biotin	µg/L	5.85	7.3	5.85	1.70	26.9	17.7
Ca-pantothenate	mg/L	2.00	7.0	2.0	1.57	2.0	16.6
Thiamine	mg/L	2.00	7.1	0.5	1.59	1.34	16.9
Pyridoxine	mg/L	3.33	7.3	1.0	1.57	1.0	16.6
Myo-inositol	mg/L	2.0	6.7	20.0	1.53	2.0	16.3
$(NH_4)_2SO_4$	g/L	2.66	7.0	2.66	1.57	3.54	17.0
KCl	g/L	0.625	7.1	0.625	1.59	0.208	16.9
H_3PO_4	g/L	0.497	7.1	0.738	1.67	0.738	18.3
$MgSO_4 \cdot 7H_2O$	g/L	0.124	6.8	0.124	1.66	0.124	17.4
$CaCl_2 \cdot 2H_2O$	g/L	0.0295	7.0	0.0442	1.59		
Yeast Extract	g/L	12.0	8.3	12.0	2.39	8.0	20.2
Cornsteep Liquor	g/L	71.0	8.2	71.0	1.51	71.0	18.6

g-ethanol/g-cells/hr, $Y_{p/s}$ = 0.43 and $Y_{x/s}$ = 0.072. The ethanol yield is equal to, but the cell mass yield is much lower than the corresponding yields ($Y_{p/s}$ = 0.43, $Y_{x/s}$ = 0.15) achieved by Cysewski (3) with high yeast extract medium. The specific productivity of the synthetic medium, however, is much greater than the q_p of 0.59 g-ethanol/g-cells/hr obtained with high yeast extract medium by Cysewski (3).

The ratio of ethanol to cell mass produced reflects both the relative stimulation of competing pathways to ethanol and cell mass and also the efficiency of energy utilization for growth. Thiamine and pyridoxine preferentially stimulated ethanol production. Biotin, pantothenate, inositol, ammonium, potassium, phosphate, yeast extract and cornsteep liquor preferentially stimulated cell mass production.

Mass transfer limitations for nutrient uptake by the cell may be significant for the concentrations of biotin and potassium found to be optimum for various fermentation parameters in Tables 7.10 and 7.11.

As evident from the complicated pathways of Figures 7.2 and 7.3, the common single limiting substrate Monod type model is not adequate to describe the non substrate growth factor effects and also the simultaneous substrate limitations of the relatively balanced base media used. The more general conceptual model proposed includes parallel and series pathways for multiply limiting substrates with competing reactions catalyzed by growth factors. Application of this conceptual model to the Figures 7.2 and 7.3 pathways helped account for the effects of growth factors as catalytic cofactors, inorganic ions as substrates and effectors, and complex components as precursors in cell mass and ethanol production. Thus, the model is useful in formulating media which can be optimized with respect to various fermentation parameters.

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Chapter 8

8. Formulation of Minimal Cost Media

8.1 Introduction

Various feed media compositions were found to be optimal for different conditions in Chapters 5, 6 and 7. These compositions are known in terms of synthetic components with and without small quantities of complex factors. The composition and the cost of potential raw materials are also known. Therefore, there should exist optimal formulations of the raw materials to minimize their total cost while satisfying given media requirements.

To find an optimal formulation, which is a linear programming problem, the Simplex method was used. This method finds the minimal cost medium formulation with linear constraints on the concentrations of the synthetic components and on the concentrations and costs of the raw materials. The component concentration constraints reflect the given component requirements as minimum values and reflect inhibitory concentrations as maximum values. This program does not trade-off raw material costs versus productivity. It must satisfy the specified component requirements with minimal excess as determined by costs and constraints on maximum concentrations.

8.2 Formulation of Linear Programming Problem

The linear programming problem can be mathematically represented as follows:

Minimize:

Medium cost =

$$c_1x_1 + c_2x_2 + \ldots + c_Nx_N$$

Subject to:

Minimum concentration for component j:

$$a_{1,j}x_1 + a_{2,j}x_2 + \ldots + a_{N,j}x_N \geq l_j$$

$$j = 1, \ldots N_l$$

Maximum concentration for component j:

$$a_{1,j}x_1 + a_{2,j}x_2 + \ldots + a_{N,j}x_N \leq u_j$$

$$j = 1, \ldots N_u$$

Maximum concentration for feed raw material i:

$$x_i \leq f_i$$

$$i = 1, \ldots N_f$$

Non-negative solutions:

$$x_i \geq 0$$

$$i = 1, \ldots N$$

where

x_i = concentration of raw material i, g/L

c_i = cost of raw material i, \$/g

l_j = minimum concentration of component j, g/L

u_j = maximum concentration of component j, g/L

$a_{i,j}$ = fraction of raw material i which is component j

N = number of raw materials

N_l = number of minimum component concentrations

N_u = number of maximum component concentrations

N_f = number of maximum raw material concentrations

The medium cost to be minimized is the sum of the products of the raw material costs and their concentrations. The concentrations of the individual active components in the medium are the sum of the products of the raw materials and their respective fractions of active components. For raw materials, such as pure vitamins, which are active components, these fractions are one. Lower concentration constraints are specified for all active components. Upper concentration constraints are specified for certain components and raw materials. Only non-negative raw material concentrations x_i, i = 1 to N, are allowed as solutions.

8.3 Bases and Assumptions

The media cost determined by the above formulation are for all raw materials supplementary to the basic sugar feedstock; sugar costs are excluded. There is no attempt to optimize the sugar source either economically in this chapter or experimentally in the other chapters. Concentrations for all supplementary raw materials are based on a glucose feed concentration of 100 g/L for a continuous stirred tank fermentor.

A list of the active components and their minimum and maximum concentration requirements are given in Table 8.1. The minimum requirements are based mainly on the experimental results with a synthetic medium for the adapted yeast described in Chapter 7. This medium was run in continuous culture at a dilution rate of 0.205/hr with an ethanol yield of 0.43 g-ethanol/g-glucose consumed. Even though the sugar was not totally utilized at this dilution rate, total sugar utilization should be achieved at a lower dilution rate with the same ethanol yield.

Table 8.1

Constraints on Concentrations of Medium Components and Raw Materials

No.	Medium Component	Minimum Conc. (g/L)	Maximum Conc. (g/L)
1	nitrogen	0.75	5.75
2	potassium	0.328	58.9
3	phosphorous	0.13	23.3
4	magnesium	0.0122	21.5
5	sulfur	0.26	
6	chloride	0.126	
7	calcium	0.00805	9.53
8	d-biotin	0.00000526	
9	Ca d-pantothenate	0.002	
10	myo-inositol	0.00126	
11	thiamine HCl	0.00134	
12	pyridoxine HCl	0.001	
13	boron	0.000875	
14	zinc	0.00113	
15	copper	0.00051	
16	manganese	0.000488	
17	aluminum	0.000236	
18	iron	0.000201	
19	cobalt	0.000105	
20	iodide	0.000382	
	Raw Material		
21	molasses (beet & cane)		200.

The maximum concentrations specified in Table 8.1 resulted in 80% reductions of cell productivity in feed component inhibition studies carried out by Maiorella (1).

Table 8.2 lists the set of potential raw materials and their approximate prices in Spring, 1982. This list contains normally available and commonly used raw materials in fermentations. Many of the required components are available from multiple sources, including chemically well defined and complex raw materials. The approximate compositions of the complex raw materials, molasses, cornsteep liquor and yeast autolysate, are reported in Table 8.3, but there are additional undefined components. The raw materials list can be easily changed as new sources develop or as prices change.

8.4 Procedure

Minimal cost media were formulated for two cases. The first case represents a medium for sugar feedstocks, such as cellulose hydrolysates, containing little or no additional nutrients. In this case all the nutrients supplementary to the sugar must be added to the medium as raw materials. The second case represents feedstocks, such as molasses or corn, containing high levels of utilizable nutrients; these require minimal supplementation.

This second case was implemented for molasses as the main sugar feedstock by assigning it a zero cost. Thus, the molasses was set at its maximum allowable concentration, which was made to correspond to 100 g/L glucose, i.e. 200 g/L molasses. The zero cost is valid because the cost being minimized is exclusive of the sugar source.

Table 8.2

Potential Raw Materials and their Unit Costs

No.	Raw Material	Unit Cost ($/gram)
1	d-biotin	8.5
2	Ca d-pantothenate	0.018
3	myo-inositol	0.0215
4	thiamine HCl	0.039
5	pyridoxine HCl	0.042
6	ammonium sulfate	0.0000716
7	potassium chloride	0.000116
8	potassium phosphate monobasic	0.000132
9	diammonium phosphate	0.000275
10	magnesium sulfate	0.000335
11	calcium chloride	0.00017
12	phosphoric acid	0.00038
13	potassium iodide	0.0205
14	boric acid	0.000608
15	zinc sulfate monohyrate	0.000441
16	copper sulfate pentahydrate	0.001
17	manganese sulfate	0.00033
18	aluminum sulfate	0.000259
19	ferrous sulfate heptahydrate	0.000143
20	cobalt sulfate monohydrate	0.00634
21	molasses (beet & cane)	
22	cornsteep liquor	0.00022
23	yeast autolysate	0.00178

Table 8.3

Composition of Complex Raw Materials

Component Number	Component	Component Fractions (g/g) for Molasses (beet & cane)	Corn Steep Liquor	Yeast Autolysate
1	Nitrogen	0.0119	0.0405	0.068
2	Potassium	0.0246	0.0243	0.0086
3	Phosphorous	0.0037	0.0059	0.015
4	Magnesium	0.00310	0.0081	
5	Sulfur	0.00144	0.45×10^{-3}	
6	Chlorine	0.0065	0.00378	
7	Calcium	0.00492	0.324×10^{-3}	0.0012
8	d-Biotin	0.74×10^{-6}	0.54×10^{-7}	2.0×10^{-6}
9	Ca-d-pantothenate	3.5×10^{-5}	1.35×10^{-5}	9.0×10^{-5}
10	Myo-inositol	3.5×10^{-3}	0.27×10^{-2}	3.0×10^{-3}
11	Thiamine HCl	0.8×10^{-6}	2.7×10^{-6}	4.8×10^{-5}
12	Pyridoxine HCl		1.08×10^{-5}	2.5×10^{-5}
13	Boron	3.0×10^{-6}	1.62×10^{-5}	
14	Zinc	7.0×10^{-5}	9.45×10^{-5}	3.87×10^{-5}
15	Copper	3.0×10^{-5}	1.35×10^{-5}	3.5×10^{-5}
16	Manganese	3.0×10^{-5}	2.7×10^{-5}	5.3×10^{-6}
17	Aluminum		$< 5.4 \times 10^{-6}$	
18	Iron	1.1×10^{-4}	1.62×10^{-4}	5.0×10^{-5}
19	Cobalt	5.0×10^{-7}		1.5×10^{-6}
20	Iodine			

A computer program was written to implement the linear program, utilizing the revised Simplex Method, which is described by Bazaraa and Jarvis (2), Hadley (3), and many others. The Simplex Method was called as Subroutine ZX3LP from the International Mathematical and Statistical Library (IMSL), version 9 (8).

8.5 Results and Discussion

The optimum medium for the case of a sugar feedstock containing no additional nutrients and requiring extensive supplementation to meet the minimum component requirements in Table 8.2 is given in Table 8.4. This table lists the raw materials, their concentrations in the optimum medium, their unit raw materials costs, and their cost contributions to the ethanol cost in $/L.

Conspicuously absent from this medium are the complex raw materials, molasses, cornsteep liquor, and yeast autolysate. Bulk chemicals (ammonium sulfate, potassium chloride, potassium phosphate monobasic, magnesium sulfate, and calcium chloride) provide the major mineral requirements. They account for $0.00714/L ethanol or 63.7% of the non-sugar raw materials cost, of which 41.5% is due to the ammonium sulfate. The growth factors are all provided as synthetic factors and account for $0.00371/L ethanol or 33.1% of the total non-sugar raw materials cost. The remaining 3.3% of this cost is for trace elements. These are probably already present as impurities in many of the raw materials or sources of industrial water. The total raw materials cost for supplementing a sugar feedstock with no nutrients is $0.0112/L ethanol or $0.0424/gal ethanol.

Table 8.4

Optimized Medium for a Sugar or Starch Feedstock Containing No Additional Nutrients

No.	Raw Material	Concentration (g/L)	Raw Material Cost ($/L ethanol)
1	d-biotin	0.00000526	0.000821
2	Ca d-pantothenate	0.002	0.000661
3	myo-inositol	0.00126	0.000498
4	thiamine HCl	0.00134	0.00096
5	pyridoxine HCl	0.001	0.000772
6	ammonium sulfate	3.54	0.00465
7	potassium chloride	0.313	0.000668
8	potassium phosphate monobasic	0.57	0.00138
9	diammonium phosphate		
10	magnesium sulfate	0.0604	0.000372
11	calcium chloride	0.0223	0.0000696
12	phosphoric acid		
13	potassium iodide	0.0005	0.000188
14	boric acid	0.005	0.0000559
15	zinc sulfate monohyrate	0.0031	0.0000252
16	copper sulfate pentahydrate	0.00201	0.0000369
17	manganese sulfate	0.00134	0.00000813
18	aluminum sulfate	0.00149	0.00000711
19	ferrous sulfate heptahydrate	0.001	0.00000263
20	cobalt sulfate monohydrate	0.000308	0.0000359
21	molasses (beet & cane)		
22	cornsteep liquor		
23	yeast autolysate		
	Total Raw Materials Cost ($/L ethanol)		0.0112

Note that the above case does not require complex medium components. As discussed in Chapter 7, the yeast may have adapted to a synthetic medium with low nutrient requirements. In earlier studies, complex factors were provided in the form of yeast extract and cornsteep liquor. The sensitivity of the ethanol cost to these complex factors have been calculated. At the actually employed levels of 1.0 g/L yeast extract and 2.0 g/L cornsteep liquor in separate media, their respective incremental costs would be $0.033/L ethanol and $0.0081/L ethanol. Since yeast autolysate is assumed equivalent to yeast extract in nutrient content, its lower cost is used. Nevertheless, this yeast autolysate cost quadruples the raw materials cost (exclusive of sugar cost) to $0.044/L ethanol. At 2.0 g/L the cornsteep liquor cost is 42% of the total raw materials cost (excluding sugars), which becomes $0.0193/L ethanol.

The second case analyzed by linear programming is the medium for a nutrients rich sugar feedstock, namely, molasses. The molasses composition assumed is given in Table 8.3 and represents the average values from concentration ranges for cane and beet molasses; mineral and vitamin data were from Harrison (4) and Solomons (6), respectively. This molasses is assumed to contain 50% utilizable sugars. Therefore, to maintain a consistent basis of 100 g/L sugar in the feed medium, 200 g/L of molasses is required. Table 8.5 shows that at this level of molasses, many of the synthetic component requirements have been eliminated. Thiamine and pyridoxine are the only synthetic growth factors required; their costs constitute 88% of the total non-sugar raw materials cost. The remaining costs are for trace elements, which may be available from other sources as discussed above. All the major mineral requirements

Table 8.5

Optimized Medium for a Nutrients Rich Feedstock - Molasses

No.	Raw Material	Concentration (g/L)	Raw Material Cost ($/L ethanol)
1	d-biotin		
2	Ca d-pantothenate		
3	myo-inositol		
4	thiamine HCl	0.00118	0.000845
5	pyridoxine HCl	0.001	0.000772
6	ammonium sulfate		
7	potassium chloride		
8	potassium phosphate monobasic		
9	diammonium phosphate		
10	magnesium sulfate		
11	calcium chloride		
12	phosphoric acid		
13	potassium iodide	0.0005	0.000188
14	boric acid	0.00157	0.0000176
15	zinc sulfate monohyrate		
16	copper sulfate pentahydrate		
17	manganese sulfate		
18	aluminum sulfate	0.00149	0.00000711
19	ferrous sulfate heptahydrate		
20	cobalt sulfate monohydrate	0.0000147	0.00000171
21	molasses (beet & cane)	200.	
22	cornsteep liquor		
23	yeast autolysate		
	Total Raw Materials Cost ($/L ethanol)		0.00183

are provided by the molasses. Excluding molasses, the total raw materials cost is only $0.00183/L ethanol. The molasses cost at $85/ton would be $0.34/L ethanol, by far the dominant cost of ethanol production from this feedstock.

The supplementary nutrients for molasses obviously are very dependent on the molasses composition, which has wide ranges. The common types of molasses, including beet, refiners cane, high test cane and blackstrap, all have different compositions. Furthermore, even the same type varies with manufacturers and with time.

Another limitation of the molasses composition is the unknown degree of assimilation of available nutrients. For example, White (5) claims only half of the phosphates in molasses can be assimilated by yeast. Also, a major fraction of the inorganic and organic nitrogen, such as betaine, in beet molasses is not utilizable (6). These claims were not incorporated into the molasses composition since the unutilizable fraction is not well established.

Hodge (7) reported that in industrial practice, batch fermentation of some blackstrap molasses requires additional ammonium sulfate, and fermentation of high test molasses requires ammonium sulfate and phosphoric acid. The need for supplementation in these cases may be because of variations in molasses composition from that assumed in this study. Furthermore, the assumed nitrogen, potassium and phosphorous contents in molasses need to be adjusted to reflect their utilizable fractions.

8.6 References for Chapter 8

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Chapter 9

9. Oxygen Requirements for Yeast Fermentation

9.1 Effects of Dissolved Oxygen Tension

9.1.1 Introduction

Oxygen serves as a nutrient, electron acceptor, and regulator of yeast metabolism. Yeasts require oxygen in low concentrations as a nutrient for biosynthesis for either fermentative or respiratory modes of growth. For respiration, additional oxygen in higher concentration is needed to serve as terminal electron acceptors in oxidative phosphorylation. Furthermore, the oxygen and sugar concentrations determine the relative extent of fermentation and respiration, thus affecting the ethanol to cell mass ratio. Dissolved oxygen is also important at various levels as an enzyme regulator in induction and repression of enzyme synthesis, such as in cytochrome induction (36,38).

With the same yeast and medium used in this study, Cysewski (1) found maximum cell mass and total ethanol productivities but minimum specific ethanol productivities at 0.7 mmHg oxygen tension for dilution rates between 0.11 to 0.3/hr. After adapting this yeast, however, 0.07 mmHg oxygen tension was optimum for total ethanol productivity. Using the same medium with a different strain of Saccharomyces cerevisiae, Nishizawa, Dunn and Bourne (2) observed maximum rather than minimum specific ethanol productivities at 10 ppb dissolved oxygen concentration, corresponding to about 0.23 mmHg oxygen tension.

The first objective of this chapter is to relate cell mass and ethanol production yields and rates to the dissolved oxygen tension. The oxygen effect was studied from approximately zero oxygen tension to the hyperbaric pressure of 346 mmHg.

9.1.2 Procedure

Studies on oxygen effects were carried out in continuous cultures with the basic apparatus setup shown in Figure 6.1. A five liter New Brunswick Scientific Company fermentor was used at a working volume of about 1.5 liter and at a dilution rate of 0.2/hr except as noted. To avoid limiting effects from other nutrients, the high yeast extract medium given in Table 1.1 was used. This medium was sterilized by autoclaving the minerals and yeast extract solution (concentrated 4.4 fold) together, filtering the 129 g/L glucose with antibiotics solution, and then mixing the two solutions to yield 100 g/L glucose in the feed.

The major analytical problem with the oxygen studies was the accurate measurement of dissolved oxygen below about 5 % of air saturation or 8.0 mmHg. This was the approximate lower sensitivity limit for the New Brunswick and Abec galvanic probes used. Thus, the dissolved oxygen levels reported by Cysewski (1) and Nishizawa et al (2) could not be reproduced since they were below the level of sensitivity of the O_2 electrode. The general trends in the reported range of optimum specific and total ethanol productivity were, nevertheless, studied by gradually reducing the oxygen inlet rate to about zero. In these cases the oxygen mole fraction of the inlet and outlet gas streams were reported to indicate a range for dissolved oxygen tension.

Another problem in studying the effect of oxygen was to isolate it from carbon dioxide effects, which are discussed in the next chapter. It was necessary to estimate the carbon dioxide production rate to adjust the inlet rates of inert gas (nitrogen or helium) and of oxygen to vary oxygen but maintain approximately constant carbon dioxide concentration. The oxygen, nitrogen, and carbon dioxide concentrations were determined by gas chromatography as described in Chapter 3.

9.1.3 Results and Discussion

The effects of very low oxygen tension are summarized in Table 9.1. These data were obtained by maintaining an approximately constant sparge rate (100 - 128 ml/min) with varying compositions of calibrated gases. Although the carbon dioxide concentration was not constant, the range of variation should not significantly affect ethanol and cell yield and ethanol specific productivity. These results indicate that the ratio of ethanol to cell mass increases as the oxygen tension is reduced to near zero. The specific ethanol productivity increased from 0.84 to 1.3 g ethanol/g cells-hr, which was also the maximum q_p achieved by Nishizawa et al (2). The ethanol yield $Y_{p/s}$ also increased while the cell yield $Y_{x/s}$ decreased as the oxygen tension was reduced. These results are consistent with the biosynthetic function of low concentrations of oxygen in lipid synthesis. As seen in Figure 7.2, the pathways to lipids and ethanol starting from glucose branch at pyruvate. Ethanol production does not require oxygen, so its rate should be unaffected or increased by the decreased rate of the competing reaction to cell mass components.

Table 9.1

Effect of Very Low Oxygen Tensions

Inlet Y_{O_2}	Outlet Y_{O_2}	Outlet Y_{CO_2}	Dry Cell Mass g/L	Ethanol g/L	Fraction Glucose Consumed	q_p hr^{-1}	$Y_{p/s}$	$Y_{x/s}$
0.0327	0.0128	0.448	8.2	35.	0.82	0.84	0.43	0.10
0.0113	0.00697	0.492	8.3	35.	0.80	0.87	0.44	0.10
0.00227	0.00159	0.414	5.1	24.	0.51	0.97	0.48	0.10
0.000223		0.230	1.6	10.0	0.21	1.2	0.47	0.076
none	none	0.291	2.4	16.	0.33	1.3	0.48	0.072

Figures 9.1 and 9.2 each show the effect of oxygen tension at low and intermediate ranges. The low range (less than 5 mmHg) dissolved oxygen tensions are based on direct probe readings but are subject to significant error. All data in these figures were obtained by varying the sparge rate of air (0 to 1760 ml/min) as the only inlet gas. The variation in Y_{CO_2} from 0.6 to 1.0 for the low range of dissolved oxygen should not have significantly affected ethanol and cell mass yields and specific ethanol productivity. The relative increase in the ratio of ethanol to cell mass production rates as reflected in specific ethanol productivity for oxygen tension approaching zero confirms the results in Table 9.1 for decreasing oxygen tension. Brown and Johnson (30) found that below 3 mmHg oxygen tension, most lipid components, especially sterol esters and unsaturated fatty acids, decreased in Saccharomyces cerevisiae grown in galactose limited continuous cultures.

For the intermediate oxygen tension range (19 to 107 mmHg), Y_{CO_2} mostly varied from 0.067 to 0.13. Over this range, many of the variables in Figures 9.1 and 9.2 were slightly affected by the CO_2 concentration as shown in the next chapter. Despite the increasing effects of CO_2 on ethanol yield and specific productivity, these variables appeared to decline gradually with respect to oxygen tension in this range. The concentration of O_2 in this range should provide sufficient oxygen for biosynthesis of lipids for membranes and other required cell components. This higher oxygen range may also, however, stimulate further lipid synthesis for storage and increase the respiration rate, resulting in decreased ethanol yield and specific productivity.

The effect of high oxygen tension is shown in Figure 9.3. The

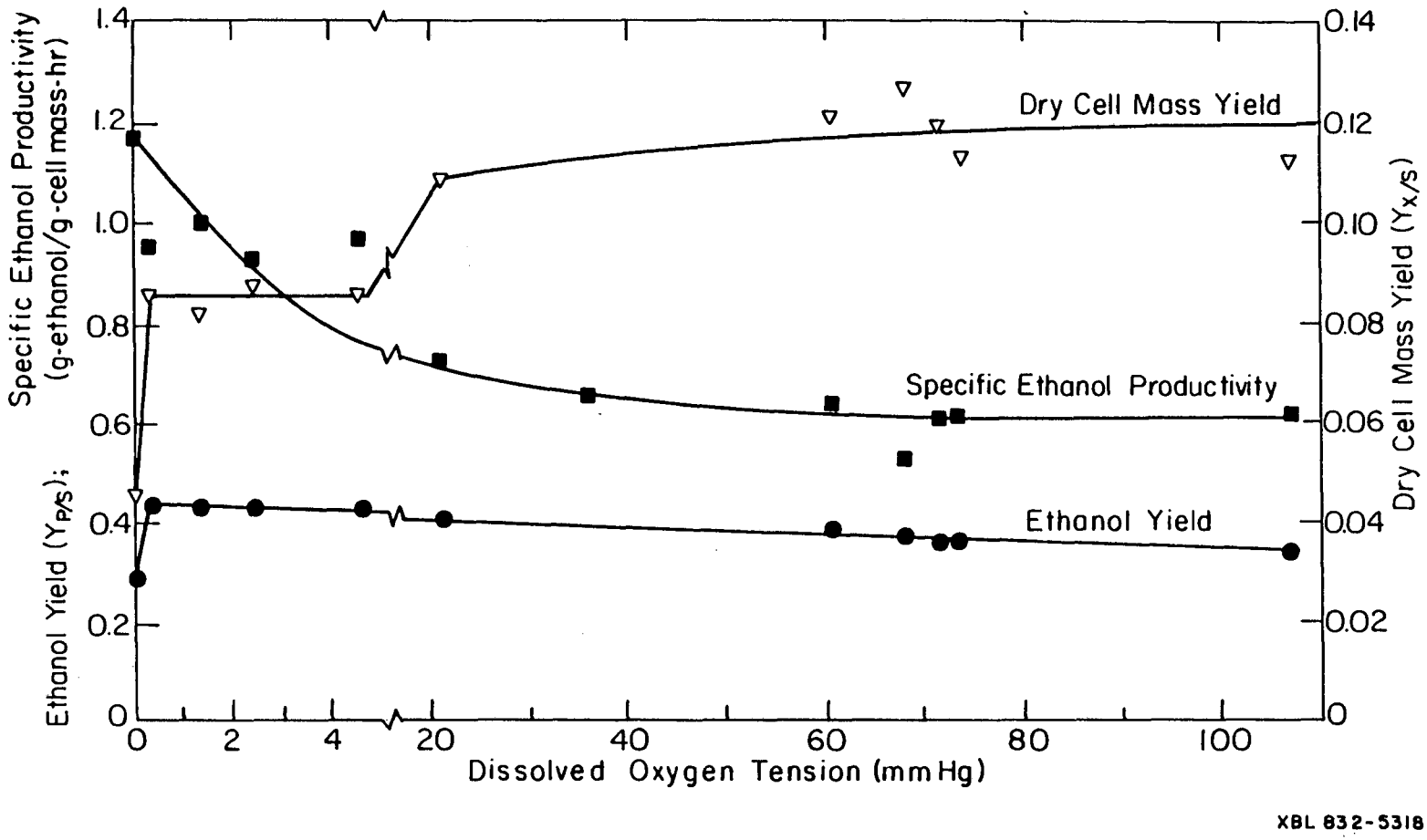

Figure 9.1 Effect of Dissolved Oxygen Tension on Specific Ethanol Productivity, Ethanol Yield, and Cell Mass Yield of Saccharomyces cerevisiae in continuous culture

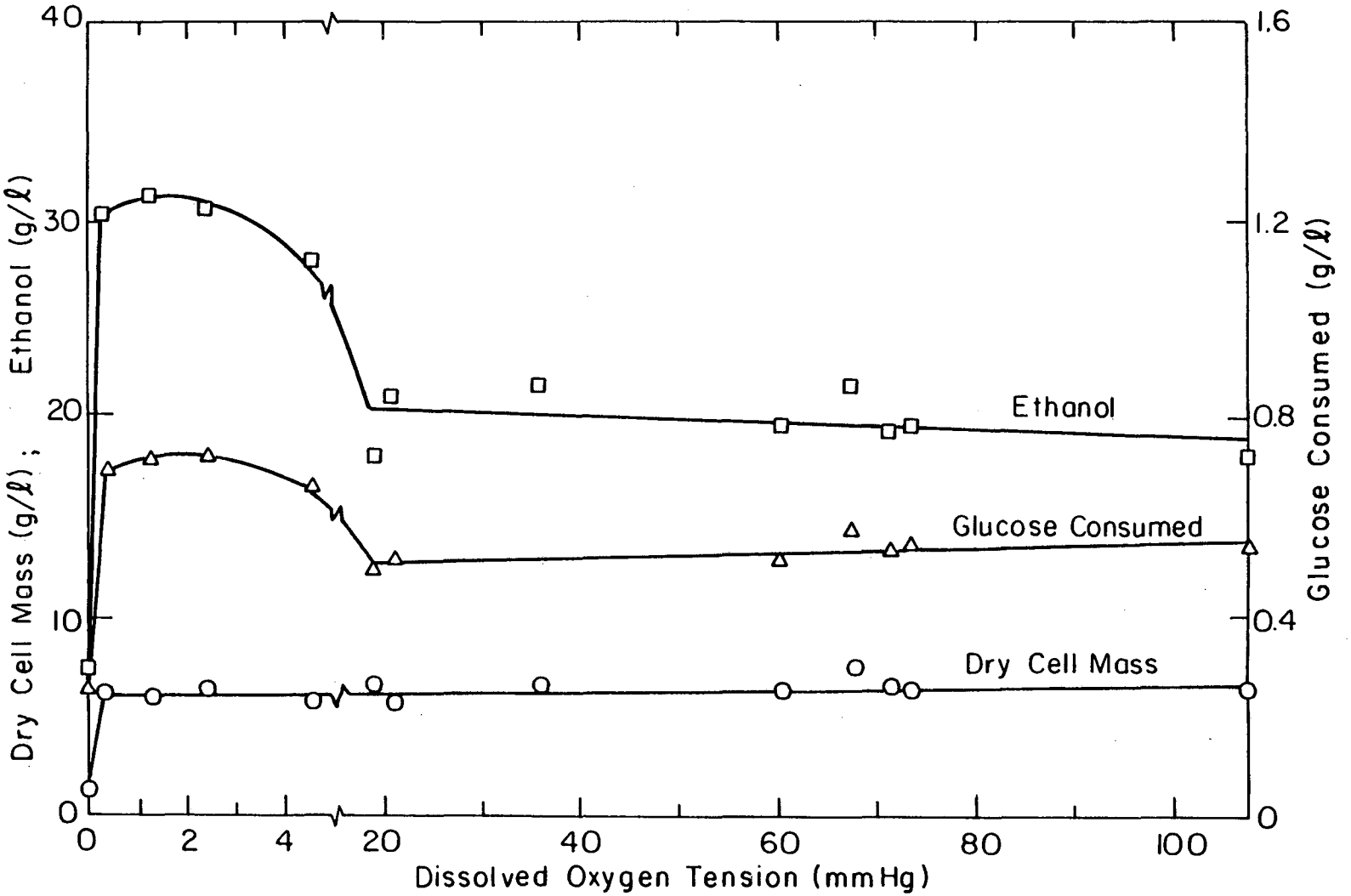

Figure 9.2 Effect of Dissolved Oxygen Tension on Concentrations of Dry Cell Mass and Ethanol and on the Fraction of Feed Glucose consumed in continuous culture of Saccharomyces cerevisiae

XBL 832-5315

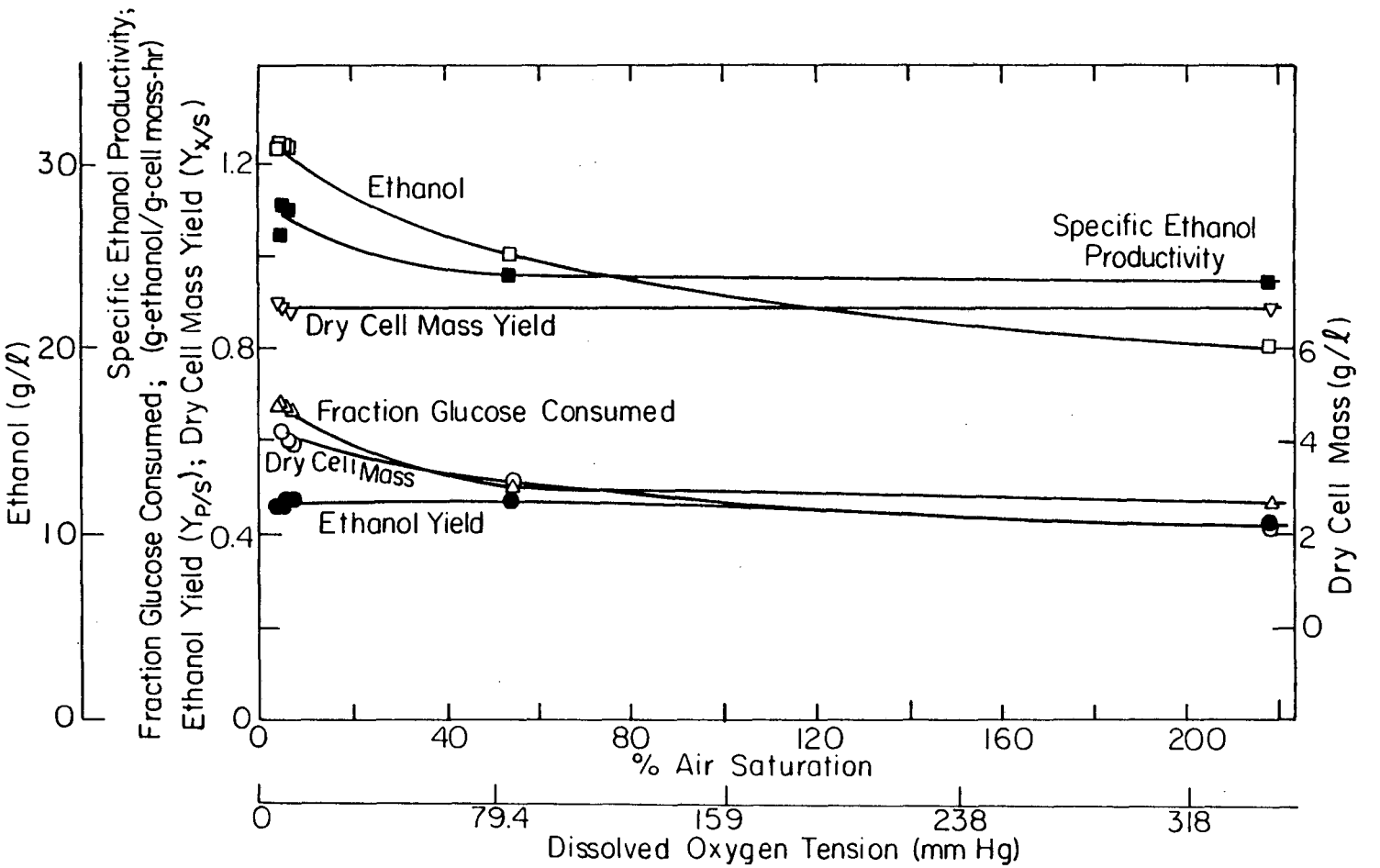

Figure 9.3 Effect of Increased Dissolved Oxygen Tension up to Hyperbaric Level on Saccharomyces cerevisiae in continuous culture

inlet gas sparge rate (120 - 149 ml/min) was approximately constant with mixtures of air and nitrogen at low oxygen tensions (less than 15 mmHg) and mixtures of pure O_2 and helium at the higher concentrations. Y_{CO_2} varied from 0.44 to 0.54 for all the data in Figure 9.3 and may have slightly affected cell mass concentration and glucose consumption. The main effect of high oxygen tension appears to be decreased glucose consumption rate with resulting decreased ethanol production rate. At 346 mmHg oxygen tension, a further decline in ethanol production rate results from the decreased ethanol yield. These results appear to be an extension of the intermediate oxygen tension results. The shift toward respiration from fermentation is further suggested by the decline in glucose consumption rate, an aspect of the Pasteur effect. However, because of the high residual glucose, this effect is counterbalanced by the catabolite repression (Crabtree) effect (7,8,9).

9.2 Oxygen Uptake Rates for Fermentation

9.2.1 Introduction

The yeast demand for oxygen for respiration can be approximately determined from the overall reaction for aerobic growth by respiration given by Harrison (3) (with coefficients rounded to three significant figures):

RESPIRATION

$$\underset{100.\ g}{0.556\ C_6H_{12}O_6} + \underset{5.10\ g}{0.300\ NH_3} + \underset{46.3\ g}{1.46\ O_2} \longrightarrow$$

$$\underset{43.2\ g}{0.300\ C_6H_{10}O_3N}\ (\text{cell mass}) + \underset{41.1\ g}{2.28\ H_2O} + \underset{67.4\ g}{1.53\ CO_2}$$

This use of oxygen for respiration is well documented and great enough

to be easily measured.

However, the biosynthetic requirement for oxygen is much less than the respiratory requirement and is less well understood. Since this is the only oxygen requirement for fermentation, it may also be considered the fermentative oxygen requirement. Haukeli and Lie (4) and Cysewski and Wilke (1) have shown that complete anaerobiosis results in very low cell and ethanol productivity. Moreover, Andreasen and Stier (5,11) showed that adding both ergosterol and unsaturated fatty acids to the medium increased cell yields significantly under anaerobiosis. This suggests that oxygen is required for the synthesis of ergosterol and unsaturated fatty acids, both important components of yeast lipid. Thusfar, this biosynthetic oxygen requirement does not appear to have been quantitatively reported. Harrison (3) does not include it in his overall reaction for yeast fermentation, but it has been included as the unknown x (in grams) in the following modification of Harrison's reaction:

FERMENTATION

$$\underset{100.\ g}{0.556\ C_6H_{12}O_6} + \underset{0.21\ g}{0.0121\ NH_3} + \underset{x}{?\ O_2} \longrightarrow$$

$$\underset{1.75\ g}{0.0121\ C_6H_{10}O_3N}\ (\text{cell mass}) + \underset{5.24\ g}{0.0570\ C_3H_8O_3} + \underset{1.42\ g}{0.0120\ C_4H_6O_4}$$

$$+ \underset{0.05\ g}{0.00071\ C_4H_{10}O} + \underset{0.21\ g}{0.0116\ H_2O} + \underset{45.3\ g}{1.03\ CO_2} + \underset{46.2\ g}{1.00\ C_2H_6O}$$

The actual yields of cell mass, glycerol, organic acids, and fusel oils vary considerably with fermentation conditions. However, these fermentation product yields are not considered in the following analyses.

The second objective of this chapter is to determine the fermentative oxygen requirement and to relate specific cell and ethanol productivities to the specific oxygen uptake rate.

9.2.2 Procedure

The stoichiometry of actual yeast fermentations can be approximated by linear combinations of Harrison's reactions for respiration and fermentation. Thus, the fermentative oxygen demand can be calculated by measuring the total oxygen uptake rate and subtracting out the oxygen uptake rate for respiratory growth. The relative extents of respiration to fermentation can be determined from the respiratory quotient, which is the ratio of the carbon dioxide evolution rate to the oxygen consumption rate.

Using a continuous culture, the carbon dioxide evolution rate was determined by measuring the total gas outlet rate from the fermentor and by measuring its CO_2 composition by gas chromatography. The oxygen consumption rate was measured by the dynamic method described by Taguchi and Humphrey (6). This method requires measuring the dissolved oxygen concentration (D.O.) in the fermentor as a function of time with a dissolved oxygen probe. After a steady state D.O. is reached, the gas inlet containing the O_2 source is turned off. At the same time the agitation rate is reduced and nitrogen is used to blanket the headspace to reduce surface aeration. The maximum slope of the D.O. decline at the inflection point yields the O_2 consumption rate. The time to reach this point from air shut-off (about 0.5 min) was calculated to be short enough to prevent the CO_2 concentration in the liquid from reaching saturation and forming bubbles, which could strip O_2 from the liquid and

contribute to the measured rate of O_2 consumption. A typical trace is shown in Figure 9.4. The initial steady D.O. level was varied by changing the agitation rate.

9.2.3 Results and Discussion

The specific oxygen consumption rate q_{O_2} as a function of the initial dissolved oxygen level is plotted in Figures 9.5 to 9.11 for dilution rates from 0.067 to 0.397/hr. For each dilution rate q_{O_2} shows approximately saturation kinetics with respect to D.O. Each of these figures represent the kinetics of an approximately constant concentration of enzymes utilizing molecular oxygen, i.e. oxygenases and oxidases. This is the concentration for cells accustomed to a predominantly fermentative mode of metabolism and thus relatively low in oxidases required for respiration. The time frame of D.O. changes for a given dilution rate is too short for further enzyme synthesis. Ferdouse, Rickard, Moss and Blanch (10) observed that mitochondria and aerobic cytochromes appeared in yeast between three to six hours after the transition from anaerobiosis to 3 micromolar dissolved oxygen concentration in the presence of only 0.02 to 1.66 mM glucose. The residual glucose concentration was sufficient at all dilution rates to exert at least this level of repression, limiting the effect of the D.O. changes to the activity of the existing enzyme system.

q_{O_2max} is the maximum specific oxygen consumption rate for a given specific growth rate with a given concentration of oxygen utilizing enzymes. Because the enzyme concentrations are low for fermentation, the q_{O_2max} values are low. These q_{O_2max} values are plotted against dilution rate in Figure 9.12. q_{O_2} for 0.067/hr dilution rate in Figure

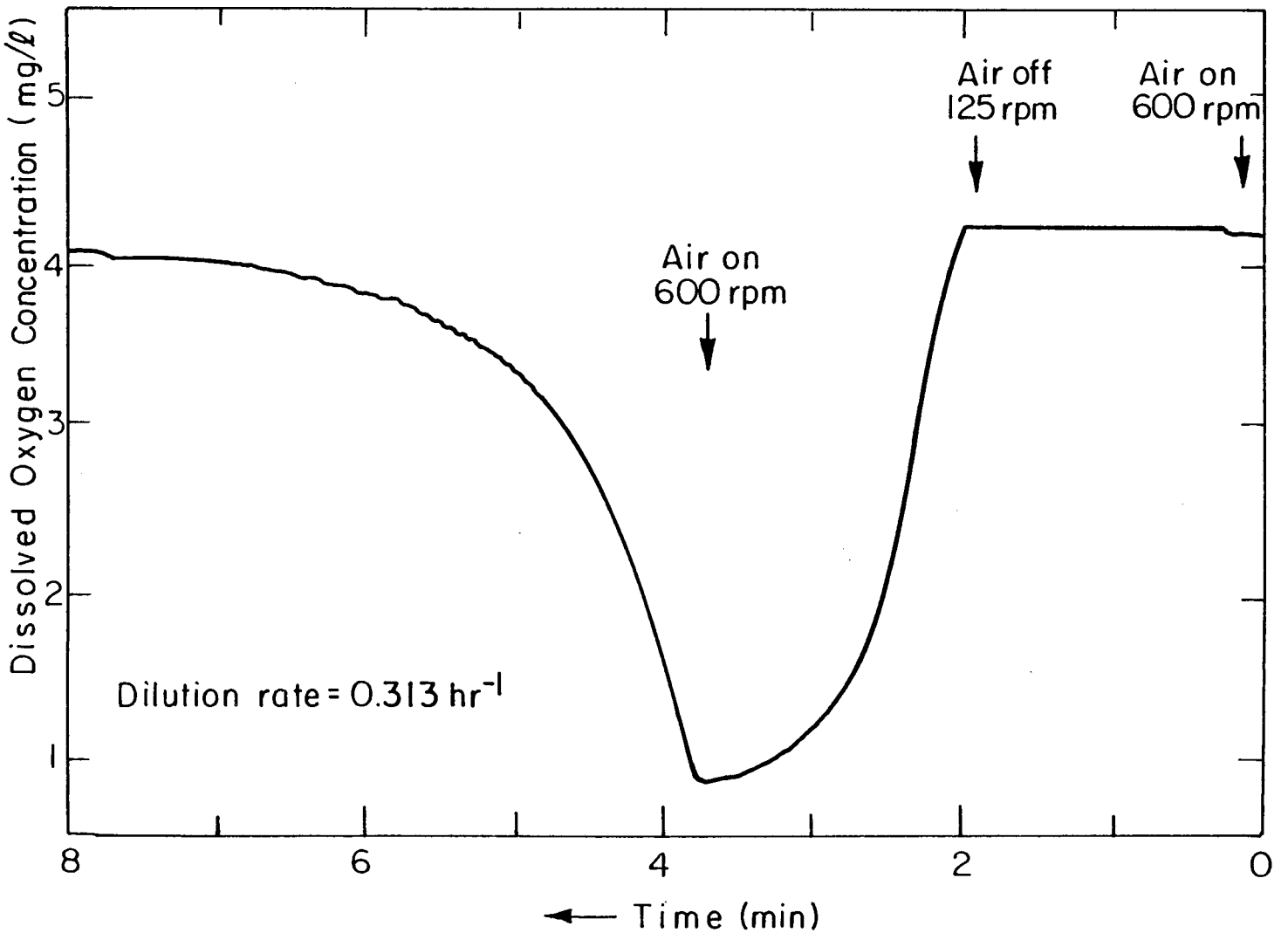

Figure 9.4 Typical Dissolved Oxygen Trace for Measuring Oxygen Consumption Rate by the Dynamic Method, (also can be used to determine the fermentor k_1a, the mass transfer coefficient times the interfacial area per volume)

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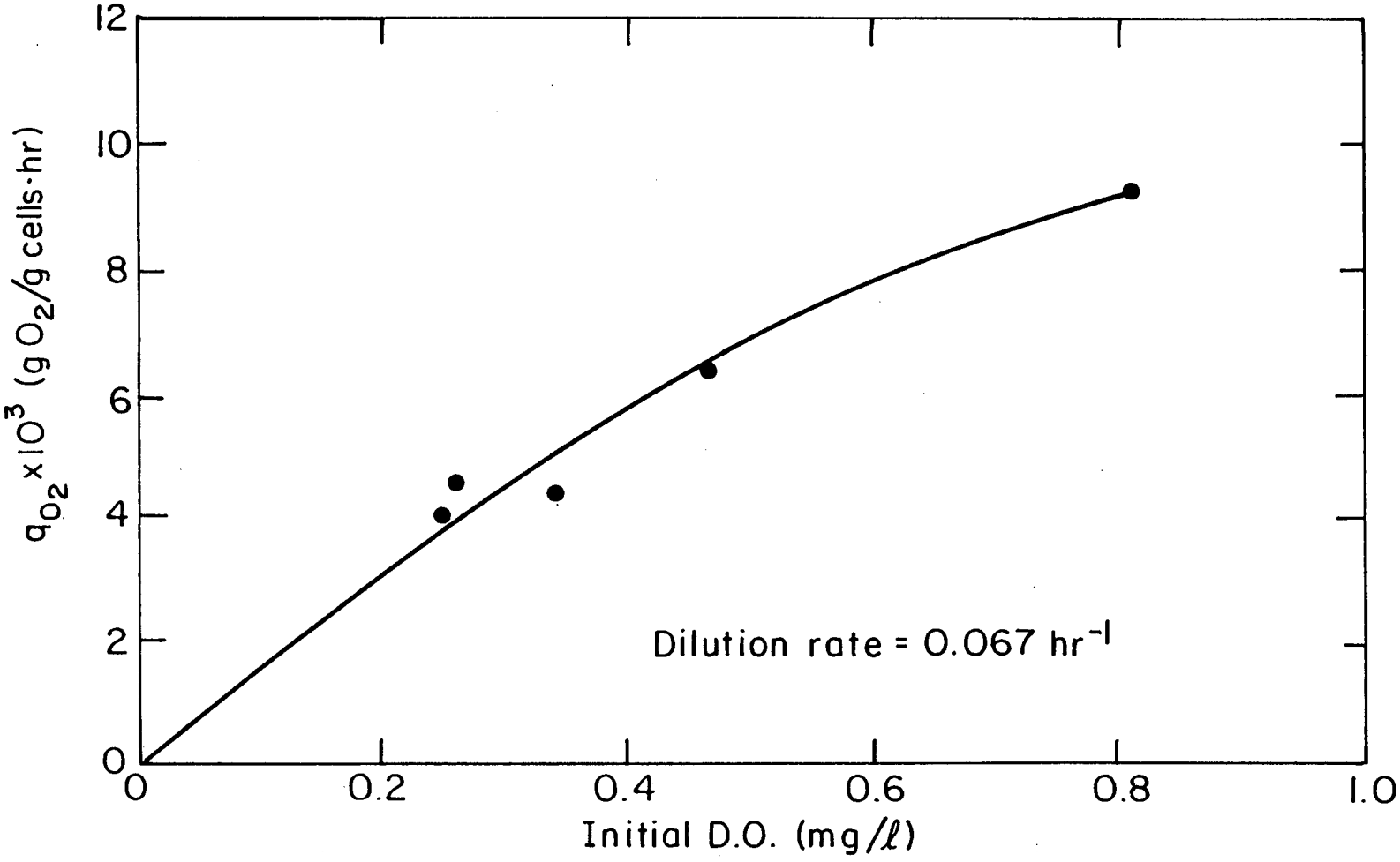

Figure 9.5 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at Dilution Rate of 0.067/hr

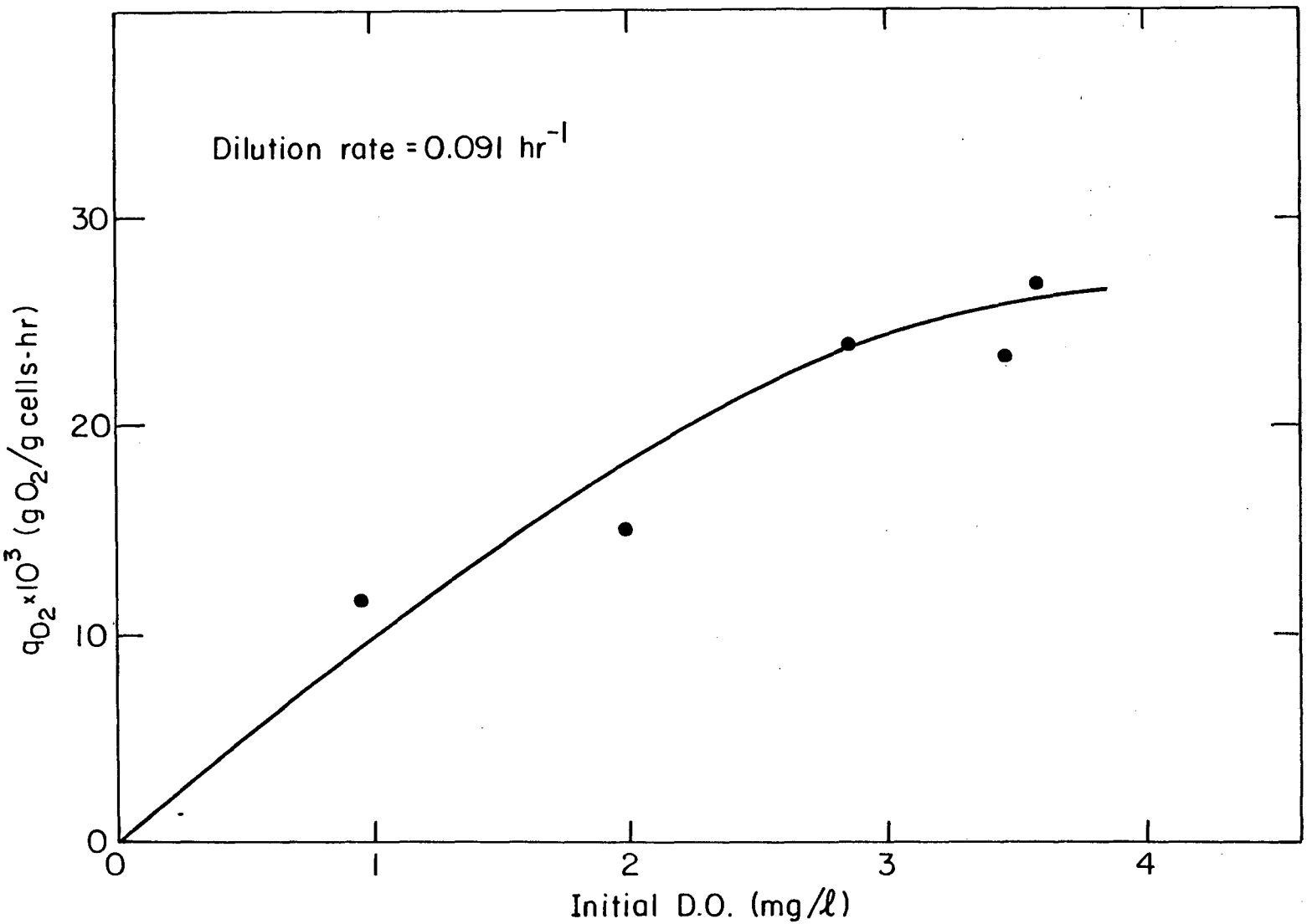

Figure 9.6 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at a Dilution Rate of 0.091/hr

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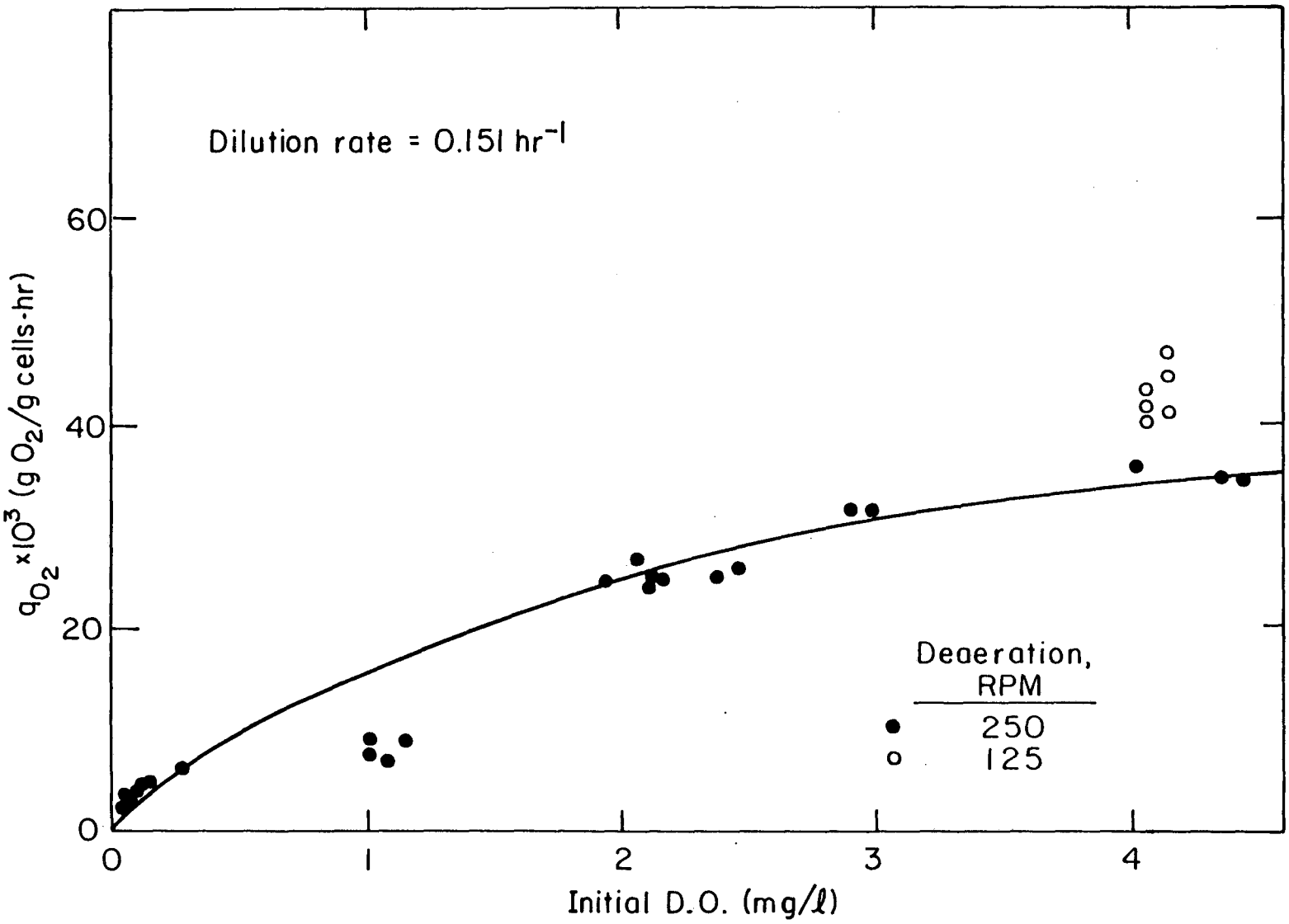

Figure 9.7 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at a Dilution Rate of 0.151/hr

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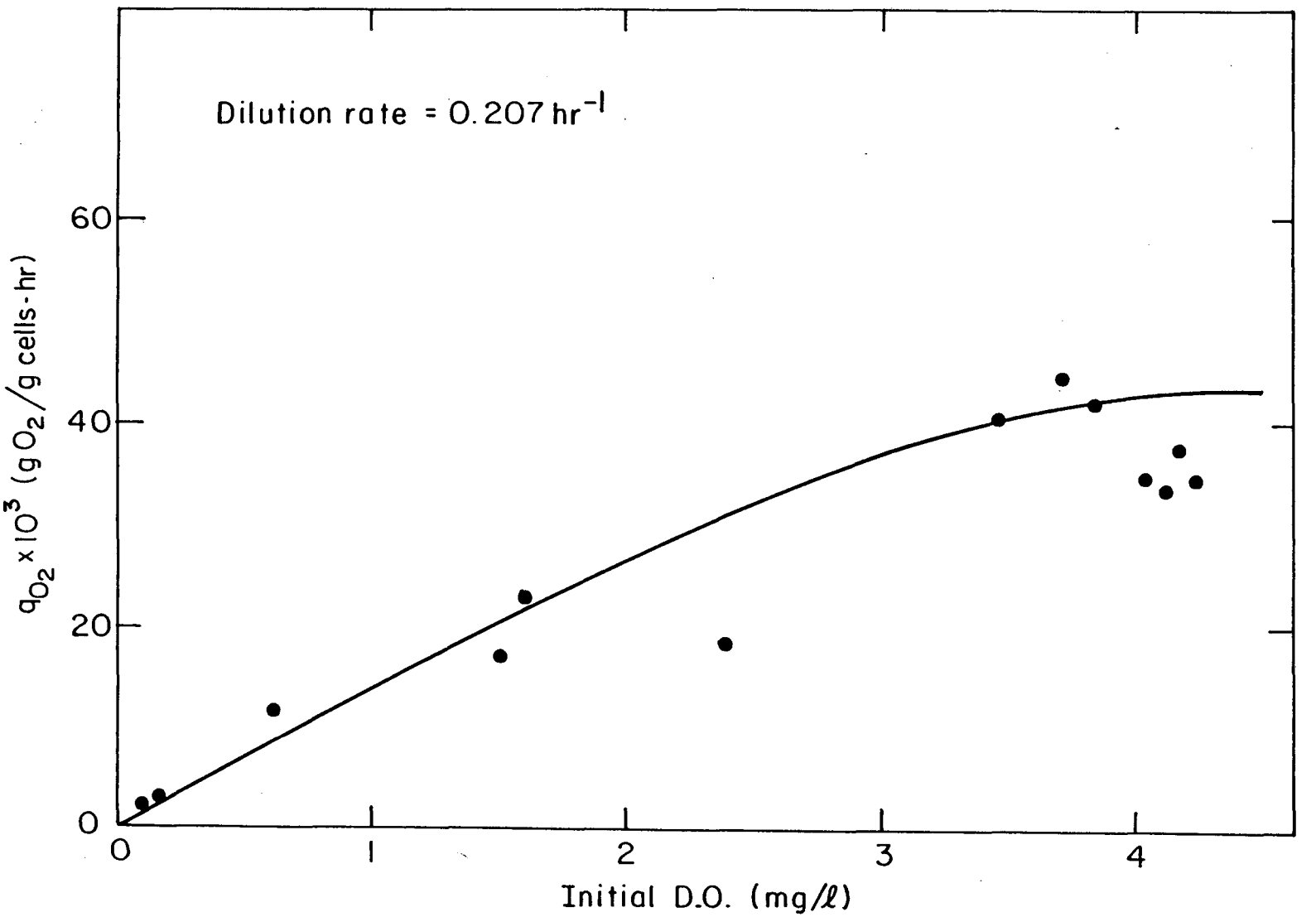

Figure 9.8 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at a Dilution Rate of 0.207/hr

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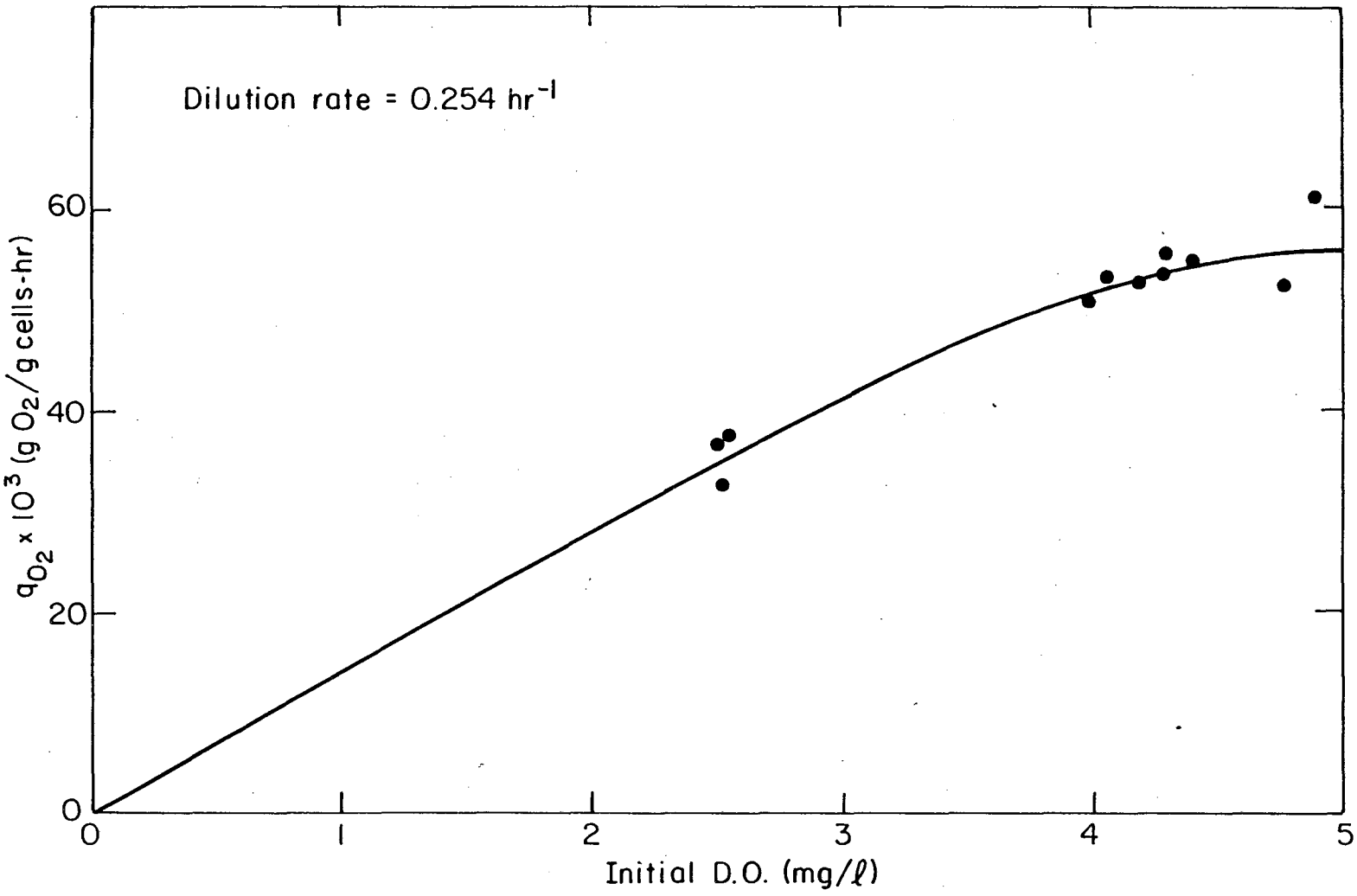

Figure 9.9 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at a Dilution Rate of 0.254/hr

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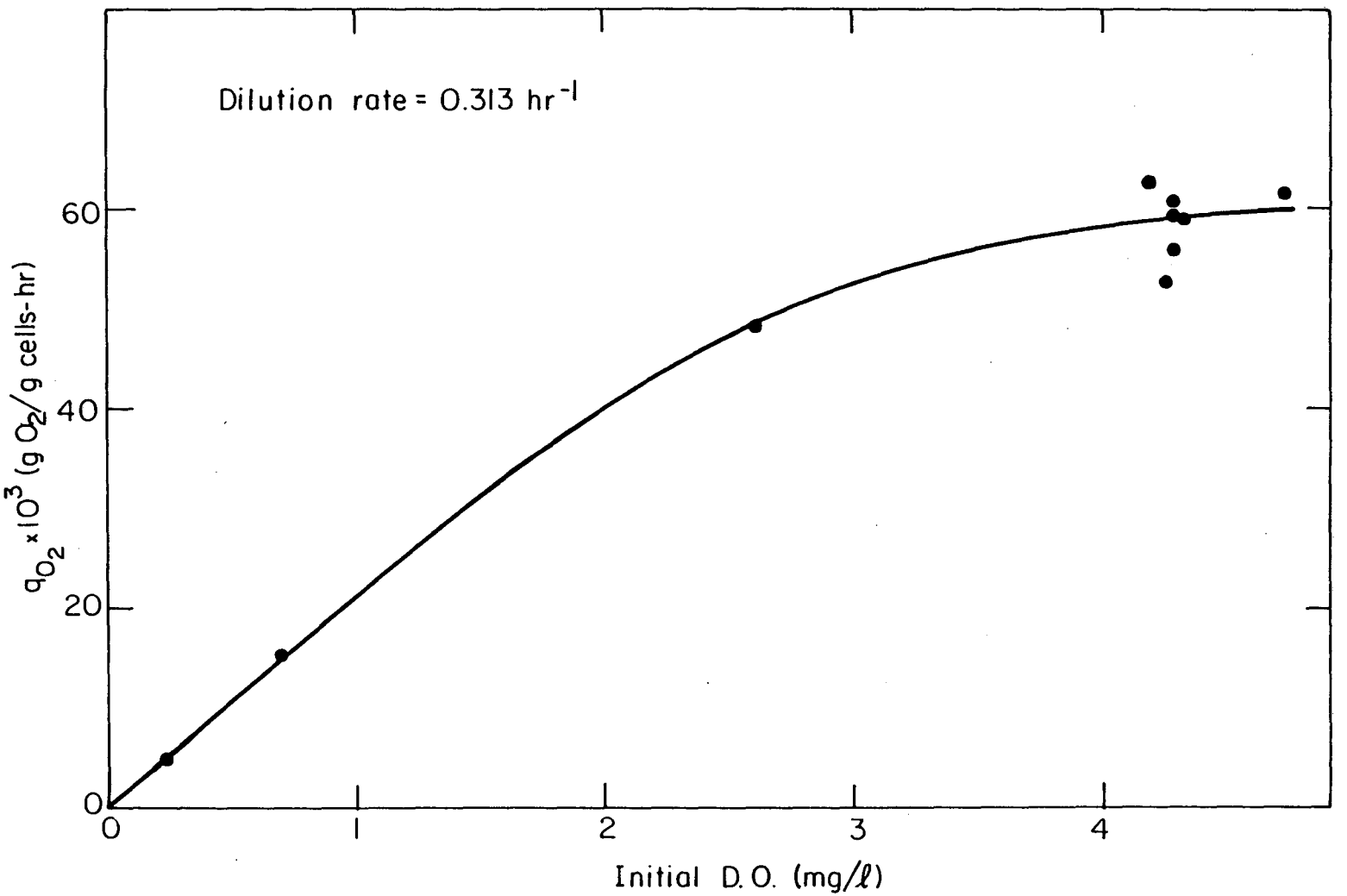

Figure 9.10 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at a Dilution Rate of 0.313/hr

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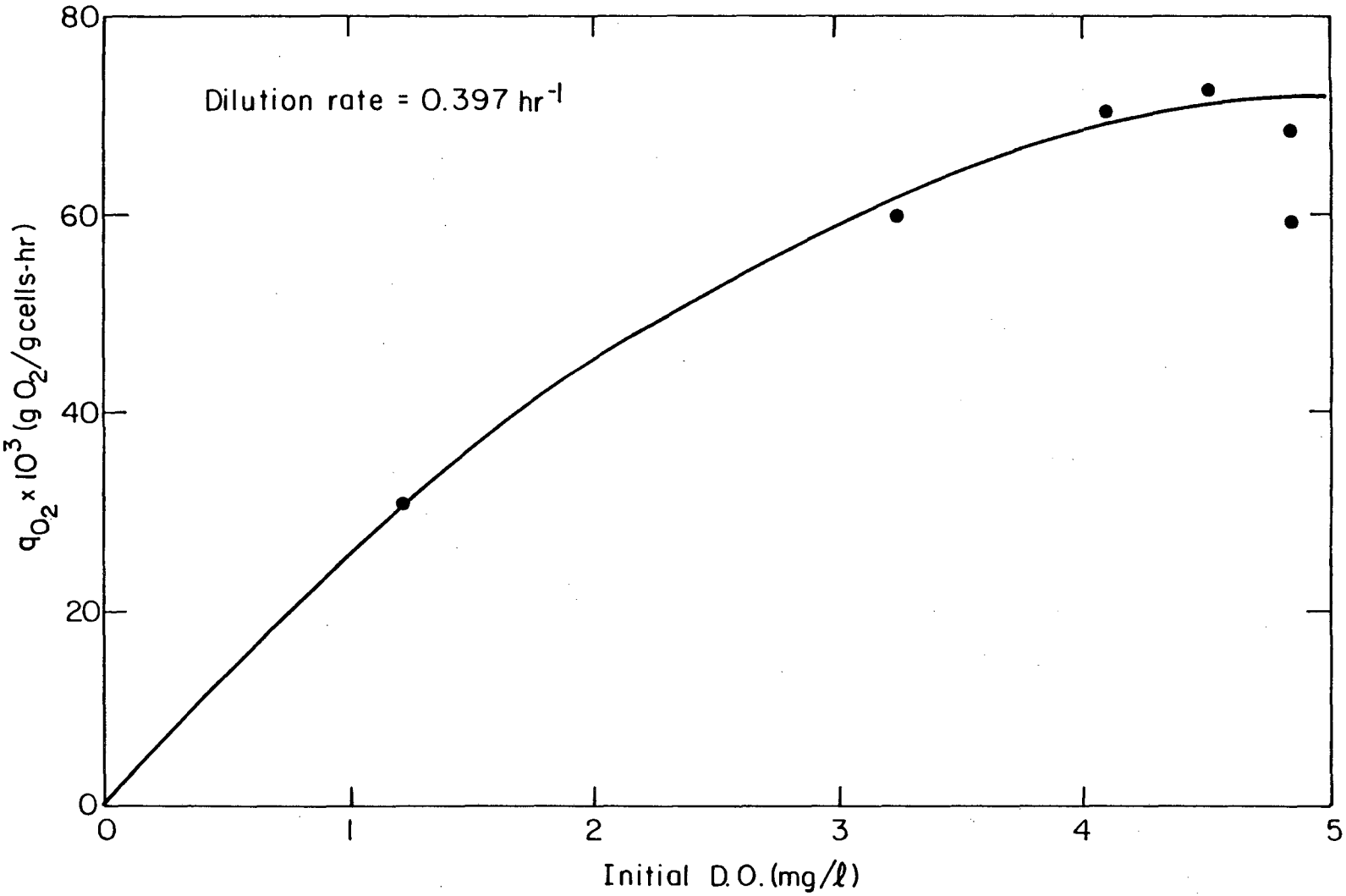

Figure 9.11 Effect of Initial Dissolved Oxygen Concentration on Specific Oxygen Consumption Rate at a Dilution Rate of 0.397/hr

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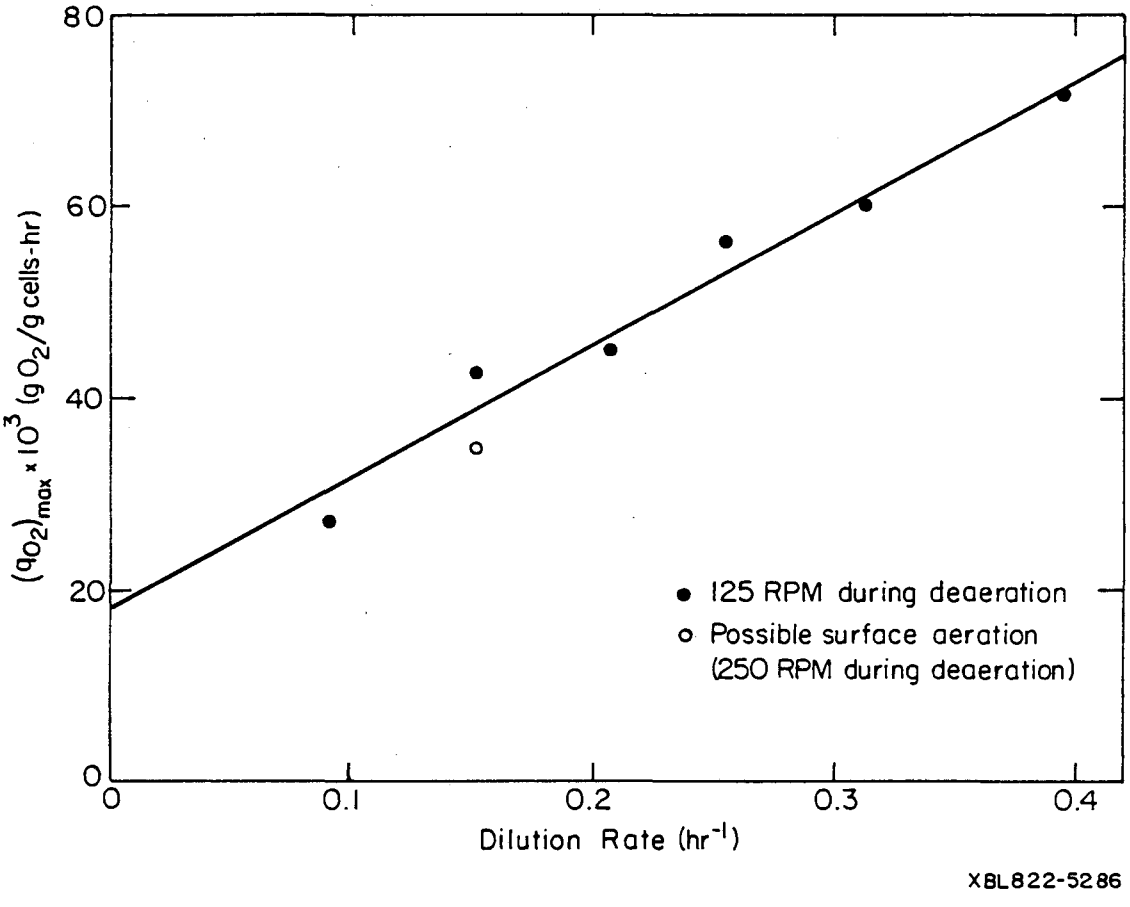

Figure 9.12 Maximum Specific Oxygen Consumption Rate as a Linear Function of Dilution Rate

9.5 does not reach a q_{O_2max} plateau and so does not appear in Figure 9.12.

The linear relationship in Figure 9.12 between q_{O_2max} and dilution rate implies that the oxygen requirement for yeast growth is constant. This relationship can be determined by linear regression to be:

$$q_{O_2max} \text{ (g } O_2\text{/g cells/hr)} = 0.018 + 0.14 \text{ dilution rate (hr}^{-1}\text{)}$$

The slope of this equation implies that 0.14 g O_2/g cells is the maximum oxygen required for growth for dilution rates up to 0.397/hr. A maintenance requirement of 0.018 g O_2/g cells-hr is determined from the intercept.

The oxygen requirement for the respiration occurring concurrently with fermentation requires determining the respiratory quotient (RQ), which is given in Figure 9.13 as a function of dilution rate. RQ is defined here as the ratio of the mass rate of carbon dioxide production (also shown in Figure 9.13) to the mass rate of oxygen utilization. The fraction of glucose fermented F can be related to RQ by multiplying Harrison's respiratory growth equation by (1-F) and his fermentation equation by F and then combining and rearranging to yield

$$F = (67.4 - 46.6\ RQ) / [22.1 - (46.6 - x)\ RQ]$$

Since x is much less than 46.6, x can be neglected in the above equation, which can then be plotted as Figure 9.14. RQ can range from 1.45 (completely respiratory growth) to infinity (completely fermentative

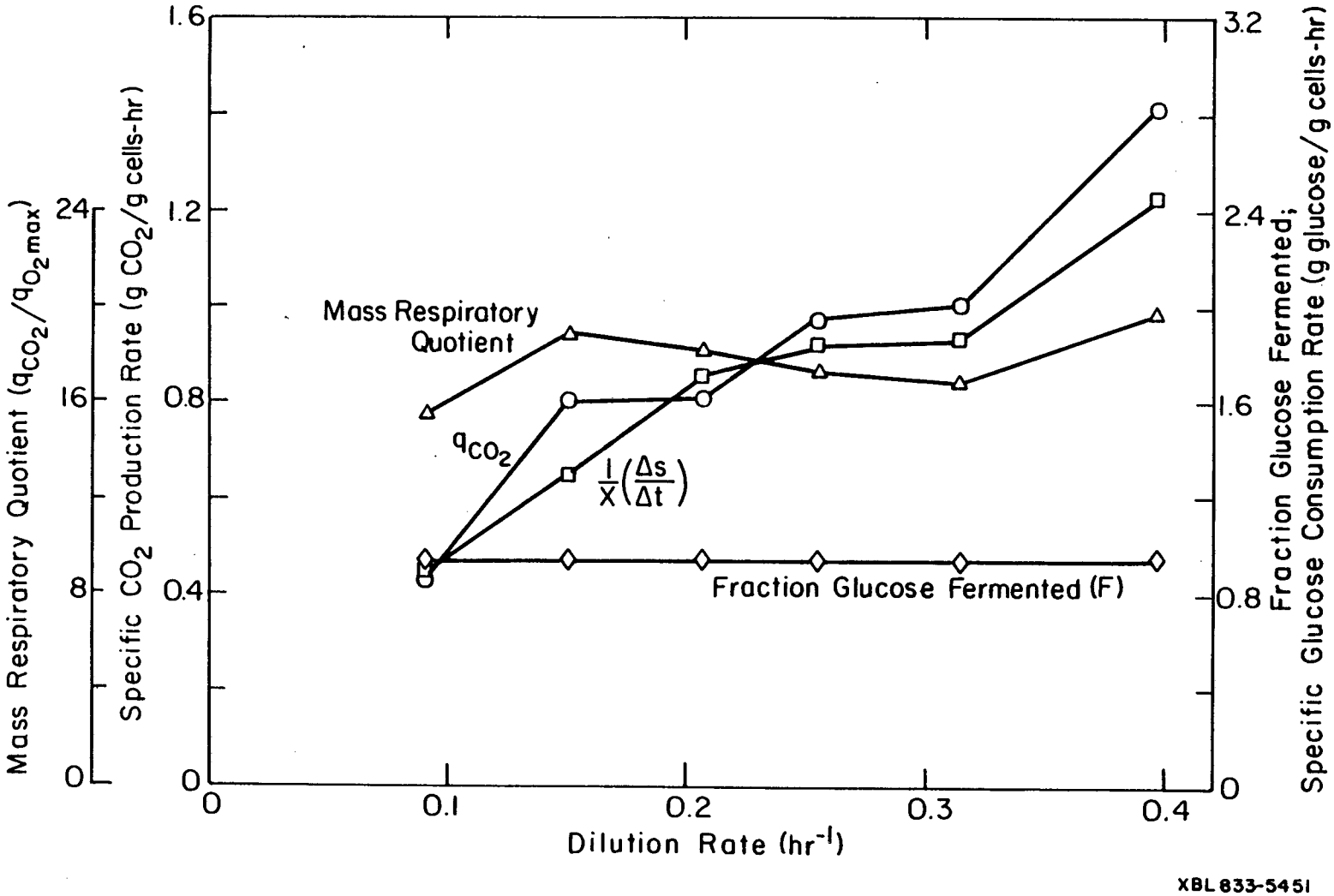

Figure 9.13 Parameters Required to Determine Respiratory Demand for Oxygen Consumption as a Function of Dilution Rate

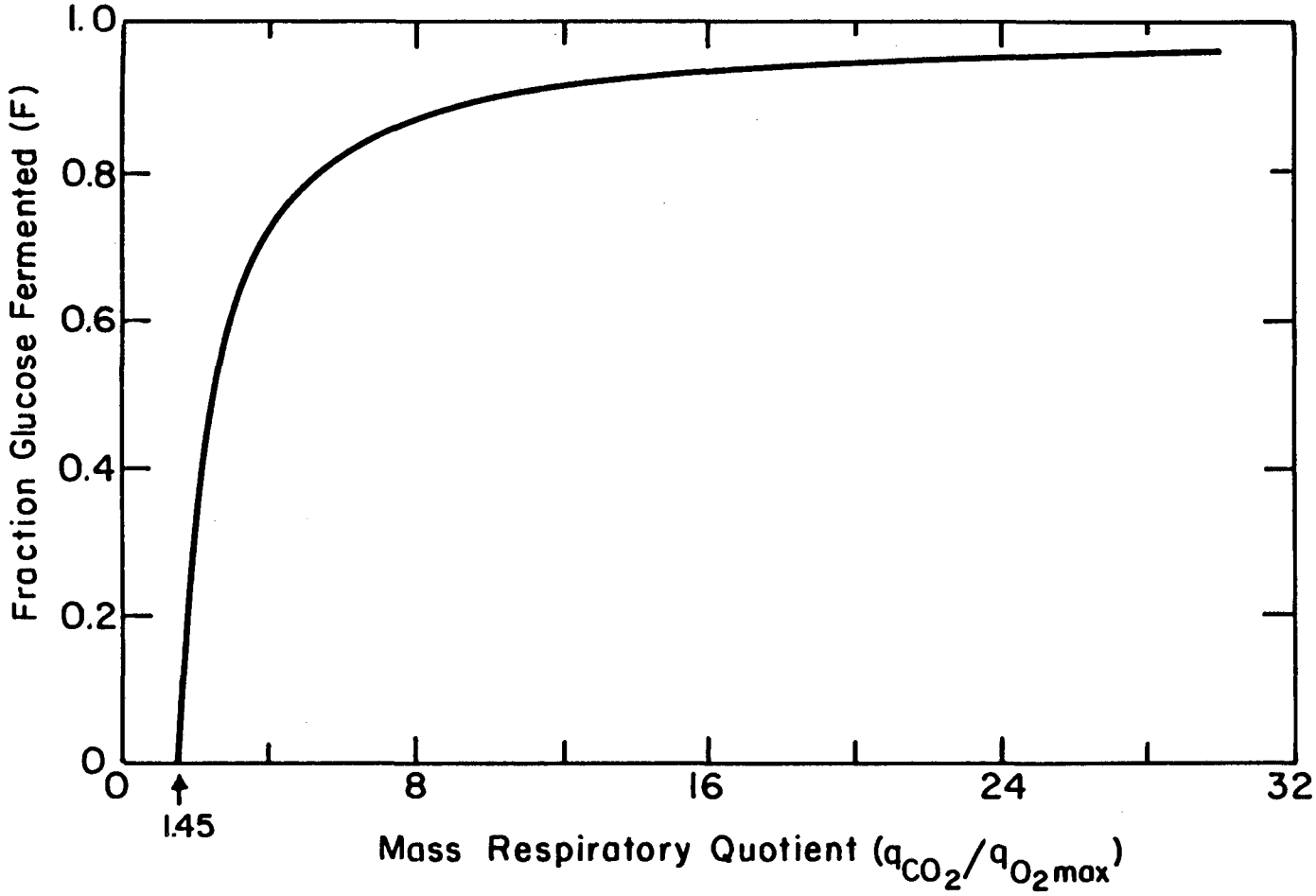

Figure 9.14 Fraction of Consumed Glucose that is Fermented as a Function of the Respiratory Quotient in Mass Units

growth). Because RQ was between 15.5 and 19.7 based on $q_{O_2 max}$, F ranged only between 0.935 to 0.949.

The respiratory oxygen demand can be determined from the following equation:

$$q_{O_2\ respiration} = (1/X)(\Delta O_2/\Delta t)_{respiration}$$
$$= (1-F)(1/X)(\Delta S/\Delta t)(\Delta O_2/\Delta S)_{respiration}$$

where (1-F) = fraction of utilized glucose respired

$(1/X)(\Delta S/\Delta t)$ = specific glucose utilization rate (g/ g cell-hr)

$(\Delta O_2/\Delta S)_{respiration}$ = grams O_2 respired per gram glucose respired

The $(\Delta O_2/\Delta S)_{respiration}$ value of (46.6/43.2) from Harrison's above respiratory growth equation was used; it agrees well with the experimental value of 1.08 given by Roels and Kossen (33). The specific glucose utilization rate as a function of dilution rate is given in Figure 9.13.

$q_{O_2\ respiration}$ with the respiratory quotient based on $q_{O_2 max}$ is plotted as a linear function of dilution rate in Figure 9.15. This is reasonable since both the respiration rate and the specific growth rate are proportional to the ATP generation rate. Linear regression yields the following equation:

$$q_{O_2\ respiration}\ (g\ O_2/g\ cells\text{-}hr) = 0.018 + 0.11\ dilution\ rate\ (hr^{-1}),$$

which implies 0.11 g O_2/ g cells is required for respiration.

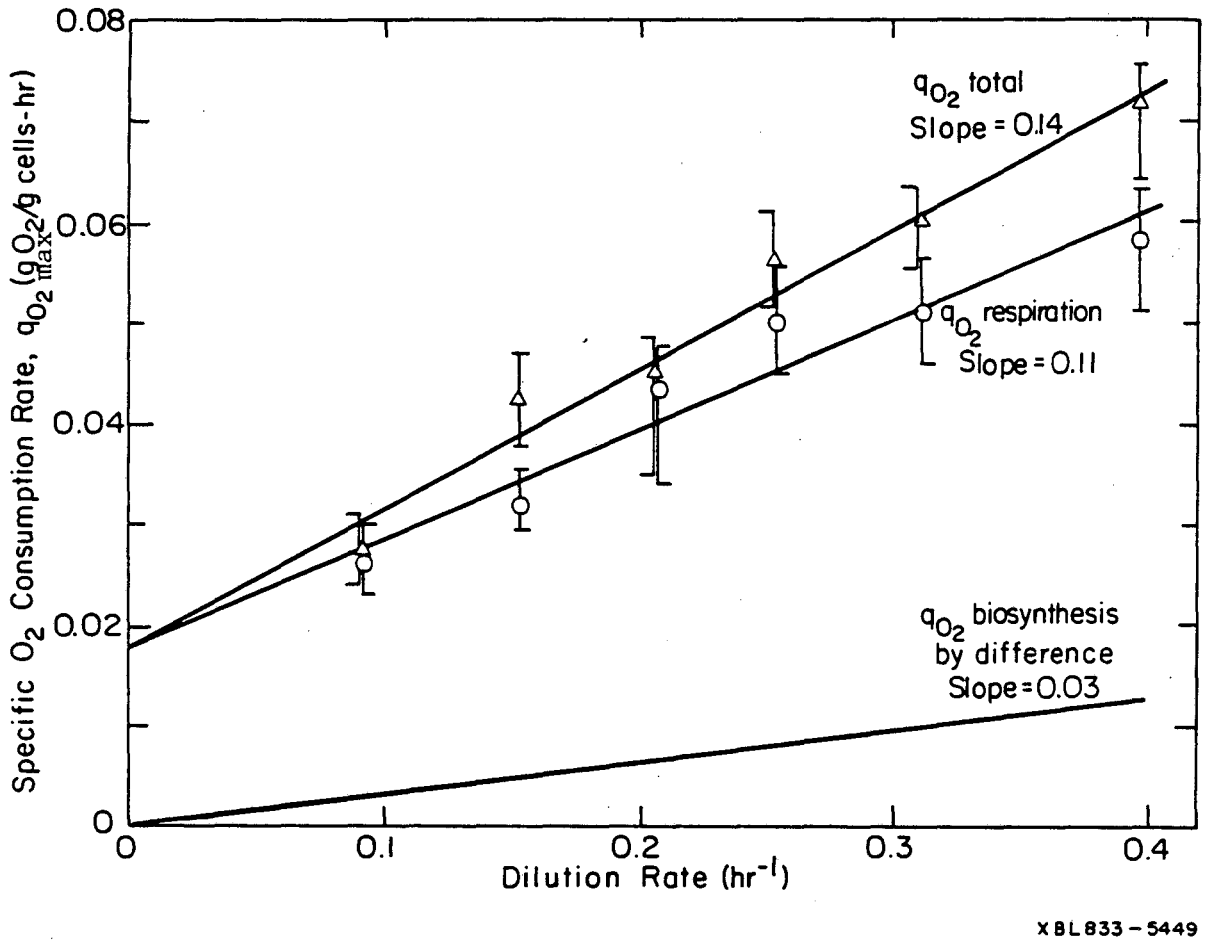

Figure 9.15 Maximum Specific Consumption Rates of Oxygen for Respiration, Biosynthesis, and Both as Functions of Dilution Rate

The $q_{O_2 max}$ values from Figure 9.12 are replotted in Figure 9.15 as $q_{O_2 total}$. The specific biosynthetic oxygen demand, $q_{O_2\ biosynthetic}$, is shown as the difference between the predicted values for $q_{O_2 total}$ and $q_{O_2 respiration}$, such that

$$q_{O_2 biosynthetic}\ (g\ O_2/\ g\ cells\text{-}hr) = 0.03\ dilution\ rate\ (hr^{-1}),$$

which implies 0.03 g O_2/ g cells is required for biosynthesis. This linear relationship is expected for cells with a constant percentage of lipid components requiring oxygen for biosynthesis with respect to specific growth rate. Furthermore, the approximately zero maintenance requirement predicted for biosynthesis is consistent with cessation of oxygen requiring cell component synthesis with a stop in total growth. Conversely, the intersection of $q_{O_2 total}$ and $q_{O_2 respiration}$ at zero dilution rate is consistent with respiration consuming the total oxygen required at zero growth to provide energy for maintenance. This maintenance oxygen requirement of 0.018 g O_2/ g cells-hr agrees closely with the value of 0.0192 g O_2/ g cells-hr given by Blanch (34).

The errors indicated by error bars in Figure 9.15 average 10 % for $q_{O_2 total}$ and 12 % for $q_{O_2 respiration}$. Since the two lines are within the error bars of all the data points, the linear models are justified. However, the errors are of the order of the difference between the two lines, and further research is required to obtain this difference more accurately.

Figure 9.16 shows a linear relationship between the specific ethanol productivity q_p and the maximum specific oxygen consumption

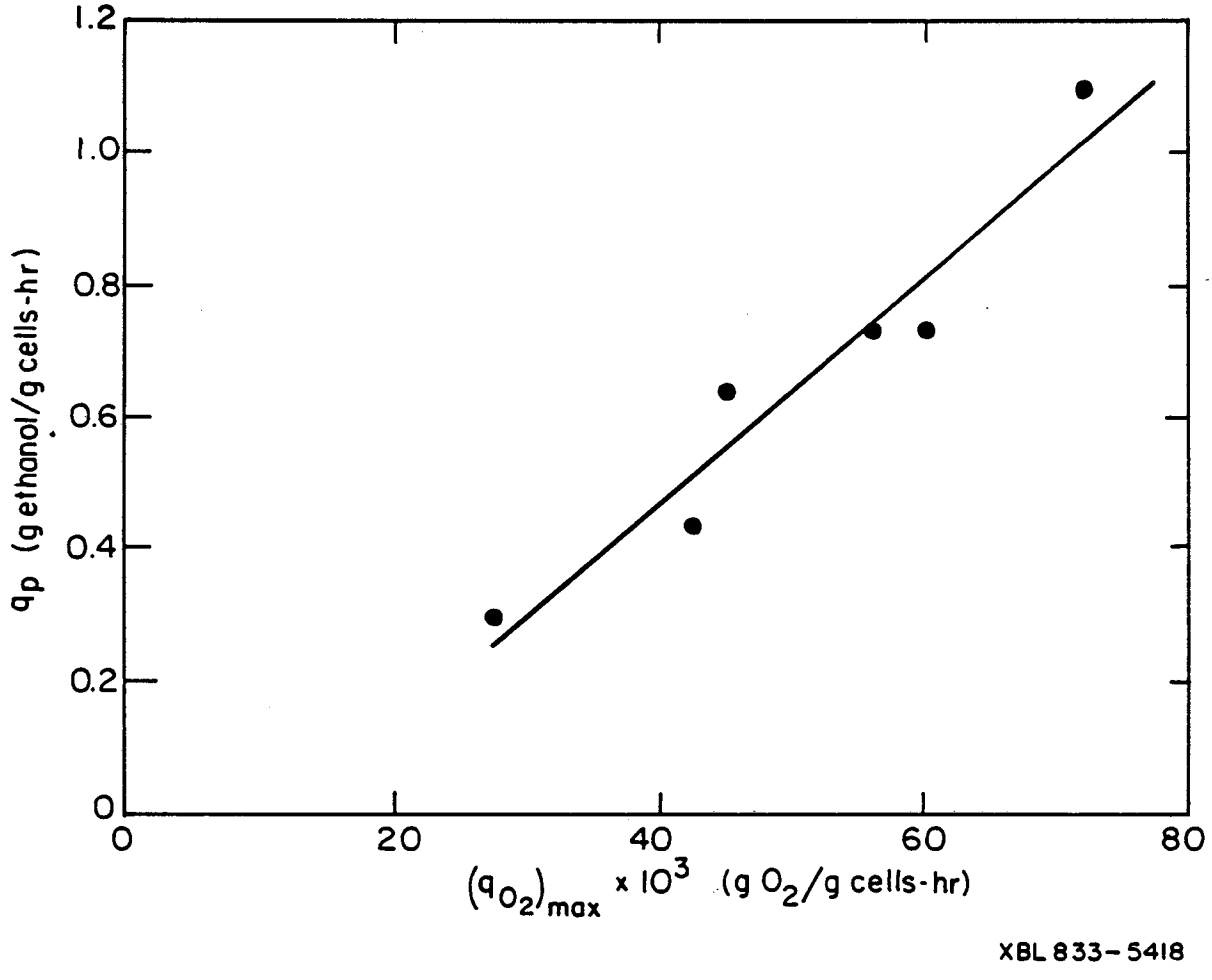

Figure 9.16 Specific Ethanol Productivity as a Function of Maximum Specific Oxygen Consumption Rate

rate. This is expected since q_p is a linear function of specific growth rate. As seen in Table 9.1 and Figures 9.1 and 9.2, high q_p requires low D.O., resulting in q_{O_2} being less than $q_{O_2 max}$. Thus, less than the saturation content of unsaturated fatty acids, ergosterol, and other oxygen requiring cell components and reduced respiration correlate with higher q_p.

The order of magnitude accuracy of the biosynthetic oxygen requirement shown in Figure 9.15 can be assessed by comparison with an estimate of the oxygen requirement for biosynthesis of unsaturated fatty acids and sterols in Saccharomyces cerevisiae. Ergosterol or other sterols and oleic acid or other unsaturated fatty acids were shown to be required for growth by this yeast under anaerobic conditions by Andreasen and Stier (5,11).

Hunter and Rose (14) reported a range of 8 to 14.4 % as the total lipid content of the dry weight of Saccharomyces cerevisiae. The bulk of this lipid is composed of triacylglycerols, phospholipids, and sterols (14). Most of the fatty acid components of triacylglycerols and phospholipids consists of palmitoleic acid and oleic acid (14, 18). The proportion of these two unsaturated acids was especially high in fermentations oxygenated in bursts as observed by Day, Webb and Martin (19) and as was done in this work. Palmitoleic and and oleic acids are derived from analogous desaturation reactions, illustrated below for oleic acid (12,14):

$$\text{Stearoyl CoA} + \text{NADPH} + H^+ + O_2 \xrightarrow{\text{oxidase}} \text{oleoyl CoA} + NADP^+ + 2H_2O$$

Further desaturation of oleic acid to linoleic acid also requires molecular oxygen and probably occurs by an analogous mechanism (14). Assuming the average oxygen requirement for fatty acid synthesis to be that for oleic acid and that fatty acids are 10 % of yeast dry weight, this requirement is 11 mg O_2 per gram cells.

Citing Shaw and Jeffries (15), Hunter and Rose (14) estimated the total sterol content of yeast to be between 0.1 to 1.0 %. Ergosterol is the principal sterol in yeast (22,28,29). Its biosynthesis from squalene is illustrated in Figure 9.17; squalene synthesis is anaerobic (29). Oxygen is first required for oxidative cyclization of squalene to lanosterol via squalene 2,3-epoxide (13,20). At least three oxygen molecules are then required for removal of each of three methyl groups from lanosterol (29,31,32). At least an eleventh oxygen molecule is required for adding the second double bond into ring B of zymosterol to form ergosterol. Assuming 0.5 % ergosterol in yeast, which is also between the 0.4 - 0.6 % content reported by Haukeli and Lie (21), the oxygen requirement for ergosterol synthesis is approximately 4.4 mg per gram cells.

The estimated oxygen requirement for unsaturated fatty acids and ergosterol totals 15.4 mg/g cells. This value is within the accuracy limits of the biosynthetic oxygen usage of 30 mg O_2/g cells given in Figure 9.15. Part of this discrepancy may also be because of the variability of cell lipid composition with respect to nutrition, aeration, and other growth conditions. Furthermore, there are unaccounted for oxygen uses, such as in the synthesis of quinone coenzymes and porphyrins (20), which are precursors to coenzyme Q and cytochromes,

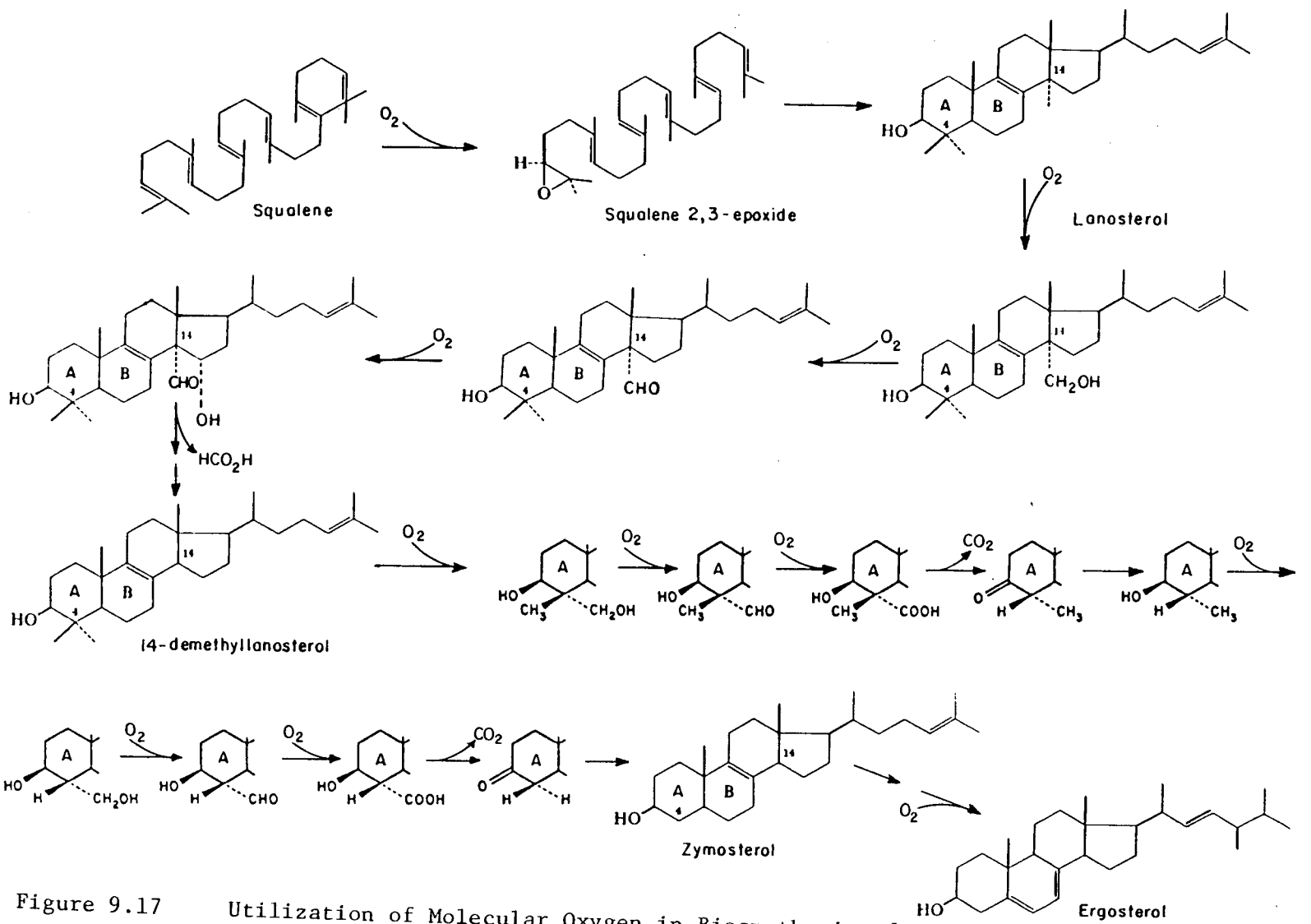

Figure 9.17 Utilization of Molecular Oxygen in Biosynthesis of Ergosterol by Yeasts

respectively.

9.3 Control of Oxygen Uptake for Fermentation

Uptake of oxygen requires transport from the medium into the cells followed by reaction within the cell. As the third objective of this chapter, the following analysis attempts to determine the relative importance of transport and reaction kinetics on the uptake rate of oxygen and to provide insight into the mechanism of oxygen transport.

The importance of mass transfer resistance can be determined from the concentration profile of O_2 from the bulk medium into the cell. In the following analyses the cells are assumed to be 4 micron spheres with a dry cell mass of 1.0×10^{-11} g/cell. The concentration drop through each component of the cell envelope can be calculated from Fick's law as follow:

$$\Delta C = q_{O_2} \, (1.0 \times 10^{-11} \text{ g/cell}) \; \Delta x \; / \; (A \cdot D)$$

where ΔC = concentration difference (g/cm^3)

q_{O_2} = specific oxygen uptake rate (g O_2/g cells-sec)

Δx = thickness of component of cell envelope (cm)

A = surface area normal to direction of flux (cm^2)

D = diffusion coefficient of O_2 (cm^2/sec)

The maximum relative drop in C, i.e. $\Delta C/C$ is obtained for the range of q_{O_2} where diffusional resistance is relatively most important. This is the initial linear region of q_{O_2} versus D.O. where the reaction kinetics of O_2 is of highest order. The maximum slope obtained for this region of

about 25 (g O_2/ g cell-hr) / (g O_2/liter) for 0.397/hr dilution rate was used in calculating $\Delta C/C$.

For the worst case of a stagnant environment, the Sherwood number = $kd/D = 2$, where k is the mass transfer coefficient, d is the cell diameter, and D is the diffusion coefficient for O_2. In this case the concentration gradient at the fluid-cell interface is of order 10^{-3} less than the bulk medium dissolved oxygen concentration.

The diffusion of O_2 through the cell wall can be modeled after bulk diffusion in porous catalysts, for which Satterfield (16) gives:

$$D_{O_2,eff} = D_{O_2} \theta / \tau$$

where $D_{O_2,eff}$ = effective diffusion coefficient for O_2 per unit cross section of porous mass (cm^2/sec)

D_{O_2} = bulk diffusion coefficient of O_2 through medium

θ = volume void fraction

τ = tortuosity factor

D_{O_2} is estimated to be 2.2×10^{-5} cm^2/sec at 35 °C, assuming a linear temperature dependence and the value reported for water by Sherwood, Pigford, and Wilke (24). Based on cell wall porosity studies reported by Arnold (23), θ should be of order 1. Satterfield (16) reports tortuosity factors approximately from 1 to 10 for catalysts; 10 will be considered for the worst case, for which $D_{O_2,eff}$ is of order 10^{-6} cm^2/sec. From Fick's law, the concentration drop across a 10 nm thick wall (8,25) is of order 10^{-4} less than the bulk medium dissolved oxygen

concentration.

The diffusion coefficient of O_2 through the plasma membrane of yeast cells has not been reported and must be estimated from membranes of other cells or model systems as given in Table 9.2. There is a wide range from 2×10^{-8} to 2×10^{-5} cm^2/sec reported for D_{O_2}, reflecting in part the varying structure and fluidity of the different systems. The corresponding concentration drop of O_2 through the membrane is approximately from 3×10^{-3} to 3×10^{-6} times the outside concentration and thus relatively small even for the lowest D_{O_2}.

Chance (37) compared the reaction rate of O_2 with cytochrome oxidase in cells to the rate with isolated mitochondria and concluded that the intracellular oxygen diffusion gradient was negligible.

Since the concentration of O_2 within the cell does not appear to be significantly different from the medium concentration, the reaction kinetics of O_2 within the cell appears to be controlling its uptake rate. Reaction control implies the kinetics observed for q_{O_2} versus D.O. in Figures 9.5 to 9.11 are for a combination of oxidases and oxygenases for respiration and biosynthesis of oxygen requiring cell components. The apparent saturation constant K_m observed was 1 to 2 mg/L, corresponding approximately to 20 to 40 mmHg O_2 tension. This apparent K_m is higher than those for most other works according to Harrison's reviews (35,36). However, there is a wide range of reported critical O_2 tension from about 0.3 to 100 mmHg. The variability can be attributed in part to changes in enzyme content with growth conditions. Simple Michaelis-Menton kinetics for a single rate limiting enzyme step do not apply if time allows for induction and repression of enzymes.

Table 9.2

Estimation of Diffusion Coefficient for O_2 through Yeast Cell Membrane

Diffusing Molecule	Membrane or Solvent	Diffusion Coefficient (cm^2/sec)	Temp. $^oC.$	Bases and Assumptions (symbols at end of table)	Comments	References
O_2	Human erythocyte	1.6×10^{-7}	25	K = 4.41 for olive oil and water, log P(cm/sec) = 1.47log K - 0.81, d = 5.0 nm, D = (P·d)/K	Authors reported that Roughton's data (40) for O_2, CO and CO_2 1.8-2.8 log units lower than predicted by this correlation, which does not include data for gases	Battino et al. (39)
O_2	Human red cell membrane	$8. \times 10^{-8}$	37	Based on diffusion and reaction model for oxygen in haemoglobin solutions with and without membranes; d = 5.0 nm	Roughton's D for O_2 much closer to Battino et al's (39) correlation than reported	Roughton (40), La Force and Fatt (41)
CH_3OH	Human red cell membrane	5.1×10^{-8}	21	d = 4.5 nm, K = 0.029, P = $(131 \pm 1.0) \times 10^{-15}$ moles/dyne/sec	CH_3OH approximation to O_2 based on same mol. wt.	Solomon (42)
CO_2	Mammalian erythocyte	1.1×10^{-7}	37	d = 5.0 nm, K = 1.6, P = 0.36 cm/sec	Approximation to D_{O_2} from averaging Roughton's (40) and Forster's (48) permeabilities	Simon and Gutknecht (43)
Nonelectrolyte	*Tollypellopsis stelligara* (alga), *Chara ceratophylla* (plant cell), human erythocyte	2×10^{-7} to 2×10^{-8}	20 - 25	d = 5.0 nm, D_{max} values assumed from correlation of log $PM^{1/2}$, $M^{1/2}$ is of order 5	Correlation include data for general nonelectrolytes but not gases	Stern (26)
Nonelectrolyte	Model oils for lipid bilayer	2×10^{-7} to 2×10^{-8}		Based on viscosity of oil comparable to lipid bilayer and Stokes-Einstein type relation	Network structure of membrane not considered	Stern (26) and Lehninger (44)

Table 9.2 (continued)

Estimation of Diffusion Coefficient for O_2 through Yeast Cell Membrane

Diffusing Molecule	Membrane or Solvent	Diffusion Coefficient (cm^2/sec)	Temp. ^oC	Bases and Assumptions (symbols at end)	Comments	References
O_2	Olive oil	$(7.46 \pm 0.79) \times 10^{-6}$	25.3	Measured	D may be high due to lack of structure in solvent; Stokes-Einstein relation does not appear valid	Davidson et al (45)
O_2	Lard	$(3.95 \pm 0.32) \times 10^{-6}$		Measured		
O_2	Dimyristoyl-L-α-phosphatidylcholine bilayer	$(8.6\text{-}17.) \times 10^{-6}$	35	Electron Spin Resonance spin exchange measurement for $D\alpha = (1.0\text{-}2.0) \times 10^{-6}$ cm^2/sec, $\alpha = 0.116$ for O_2 in olive oil	$D\alpha$ is maximum along center of bilayer and minimum along surfaces; α is presently being determined for lipid bilayers by Plachy (47)	Windrem and Plachy (46), Battino et al. (39)
CO_2	Egg lecithin/ cholesterol/ decane bilayer	2.2×10^{-7}		K = 0.8, d = 5.0 nm, P = 0.35 cm/sec	K found to be about 50 % less in lipid bilayers than in model solvents	Simon and Gutknecht (43)

Symbols:

K = partition coefficient for membrane or solvent to water
P = permeability coefficient
d = diffusion distance (membrane thickness)
D = diffusion coefficient
M = molecular weight
α = Bunsen coefficient of solubility for O_2

Since transport does not appear to be limiting the specific O_2 uptake rate, the mechanism of simple diffusion is adequate for the rates of O_2 reaction observed for fermentative growth. The active transport mechanism presumed by Mukhopadhyay and Ghose (38) for O_2 transport through cell membrane is, therefore, not required.

Furthermore, the simple diffusion mechanism and the negligible dissolved oxygen concentration difference between the medium and internal cell imply that the reaction controlled oxygen flux rate to the cell is determined by the medium dissolved oxygen. Hence, the medium D.O. is the control variable, but the resulting O_2 flux determines the rates of other internal reactions affecting cell mass and ethanol production rates and yields.

9.4 Conclusions

The specific ethanol productivity in continuous culture appears to be maximum for essentially zero oxygen tension as achieved by nitrogen sparging. Increasing the oxygen tension decreased the specific ethanol productivity but did not produce the slight minimum point observed by Cysewski (1). Otherwise, the trends in q_p are similar. At low concentrations, oxygen is required for biosynthesis; the initial increase in oxygen tension from zero provides this oxygen requirement and may also induce cytochrome synthesis to lower q_p. However, at intermediate and hyperbaric tensions, the increased oxygen tension shifts the yeast metabolism to more respiration and less fermentation with decreased glucose consumption rate.

The specific uptake rate of O_2 shows approximately saturation

kinetics with respect to the medium dissolved oxygen concentration. The maximum specific uptake rate q_{O_2max} for each dilution rate is a linear function of the dilution rate for chemostat cultures, implying a constant oxygen requirement per gram yeast. Since the specific ethanol productivity q_p is a linear function of specific growth rate, q_p is also a linear function of q_{O_2max}.

The fermentative oxygen requirement was concluded to be of order 30 mg O_2/g cells. Approximately 11 mg O_2/g cells can be attributed to desaturation of fatty acids, mainly to oleic and palmitoleic acids. Assuming 11 O_2 molecules per ergosterol, about 4.4 mg O_2/g cells is required for ergosterol synthesis. Therefore, approximately half of the total fermentative O_2 consumption can be accounted for. The discrepancy in the total O_2 requirement is probably due to the variability of lipid composition and concentration and to unaccounted for oxygen usages.

Mass transfer of O_2 from the medium into cells in predominantly fermentative growth can be achieved by simple diffusion with negligible concentration drop. Therefore, internal cell reactions control the steady state uptake rate of oxygen, which affects the mode and rates of cell metabolism. This flux rate of O_2 in turn is controlled by the medium dissolved oxygen concentration.

9.5 References for Chapter 9

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Chapter 10

10. Environmental Factors in Yeast Fermentation

10.1 Temperature

Cysewski (1) determined the optimum temperature for maximum specific growth rate and specific ethanol productivity in batch culture to be 35 °C. for Saccharomyces cerevisiae, ATCC 4126. Maiorella (2) found an Arrhenius type relationship between cell productivity and temperature in continuous culture with maximum cell productivity at 32 °C. for the same organism. The American Type Culture Collection (3) reported this yeast has a temperature optimum from 39.5 to 41.5 °C. and that it has been used in the Amylo process for ethanol production.

To determine if the optimum temperature for the yeast in this study changed through adaptation to lower temperatures, a lyophilized culture was obtained from the American Type Culture Collection to study temperature effects in batch shake flask cultures. The composition of the medium used is given in Table 1.1 except antifoam was omitted.

As seen in Figure 10.1, the maximum specific growth rate for this new culture was highest at 35 °C. at a value of 0.44/hr. These results agree well with those of Cysewski (1). Thus, the temperature optimum for specific growth rate did not appear affected by adaptation by Cysewski's or this work. Over the range 28 to 35 °C. the final ethanol yield determined in the stationary phase was not significantly affected.

10.2 By-product Inhibition

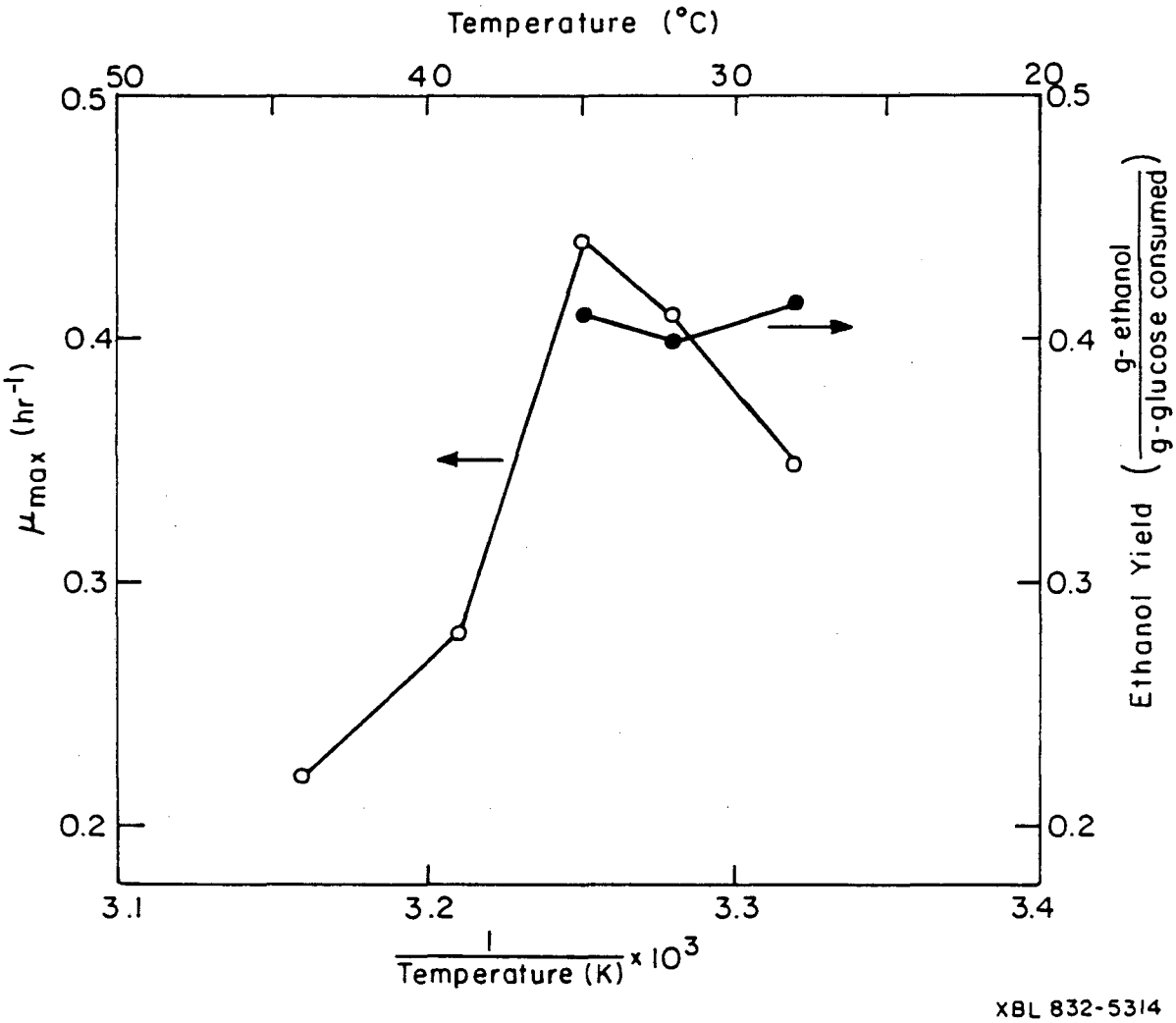

Figure 10.1 Effect of Temperature on Maximum Specific Growth Rate and Ethanol Yield $Y_{p/s}$ of *Saccharomyces cerevisiae* ATTC 4126 in Shake Flask Cultures

10.2.1 CO_2 Effects

The likelihood of CO_2 effects on yeast growth and ethanol production was noticed during the studies of oxygen effects on yeast. For example, sparging previously unaerated continuous cultures with nitrogen increased cell and ethanol productivities. The total inlet gas sparge rate, in addition to the O_2 content, appeared to be a significant factor in cases where O_2 or air was sparged. Therefore, the CO_2 and O_2 effects need to be separately determined. The CO_2 effect was studied by holding the O_2 mole fraction in the headspace (Y_{O_2}) within the range 0.016 to 0.035, while varying the mole fraction of CO_2 in the headspace (Y_{CO_2}). The high yeast extract concentration medium given in Table 1.1 was used without the antifoam.

Figure 10.2 shows the effect of varying Y_{CO_2} over the range 0.05 to 0.90. Figure 10.3 shows that Y_{O_2} over the range 0.016 to 0.035 did not significantly affect the fermentation parameters. The major effects of CO_2 appear to be over the Y_{CO_2} range from 0.05 to about 0.5 with decreasing glucose utilization and cell mass yield, but increasing ethanol yield and ethanol specific productivity.

The inhibitory effect of CO_2 on cell mass yield is consistent with the decreased activity of enzymes in amino acid and protein synthesis occurring with elevated CO_2 partial pressure, as reported by Jones and Greenfield (5) with reference to Pekur (4). Jones and Greenfield (5) also suggest a possible basis for carbon dioxide inhibition of growth as follows: increased CO_2 partial pressure increases the degree of unsaturation in the fatty acids of cell membranes and thus alters the membrane fluidity, resulting in inhibition of specific transport permeases

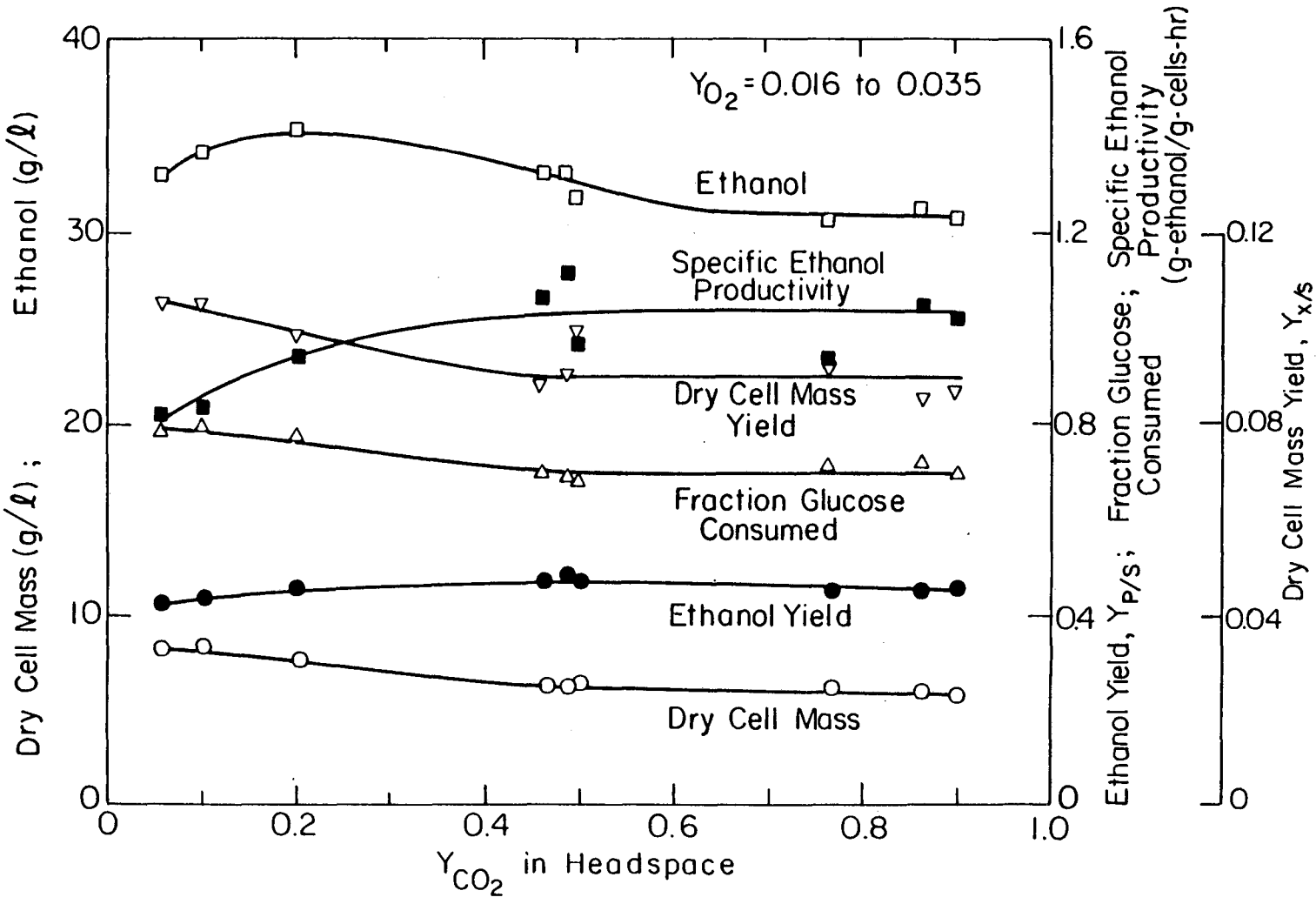

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Figure 10.2 Effect of the Carbon Dioxide Mole Fraction in the Fermentor Headspace on the Continuous Culture of Saccharomyces cerevisiae

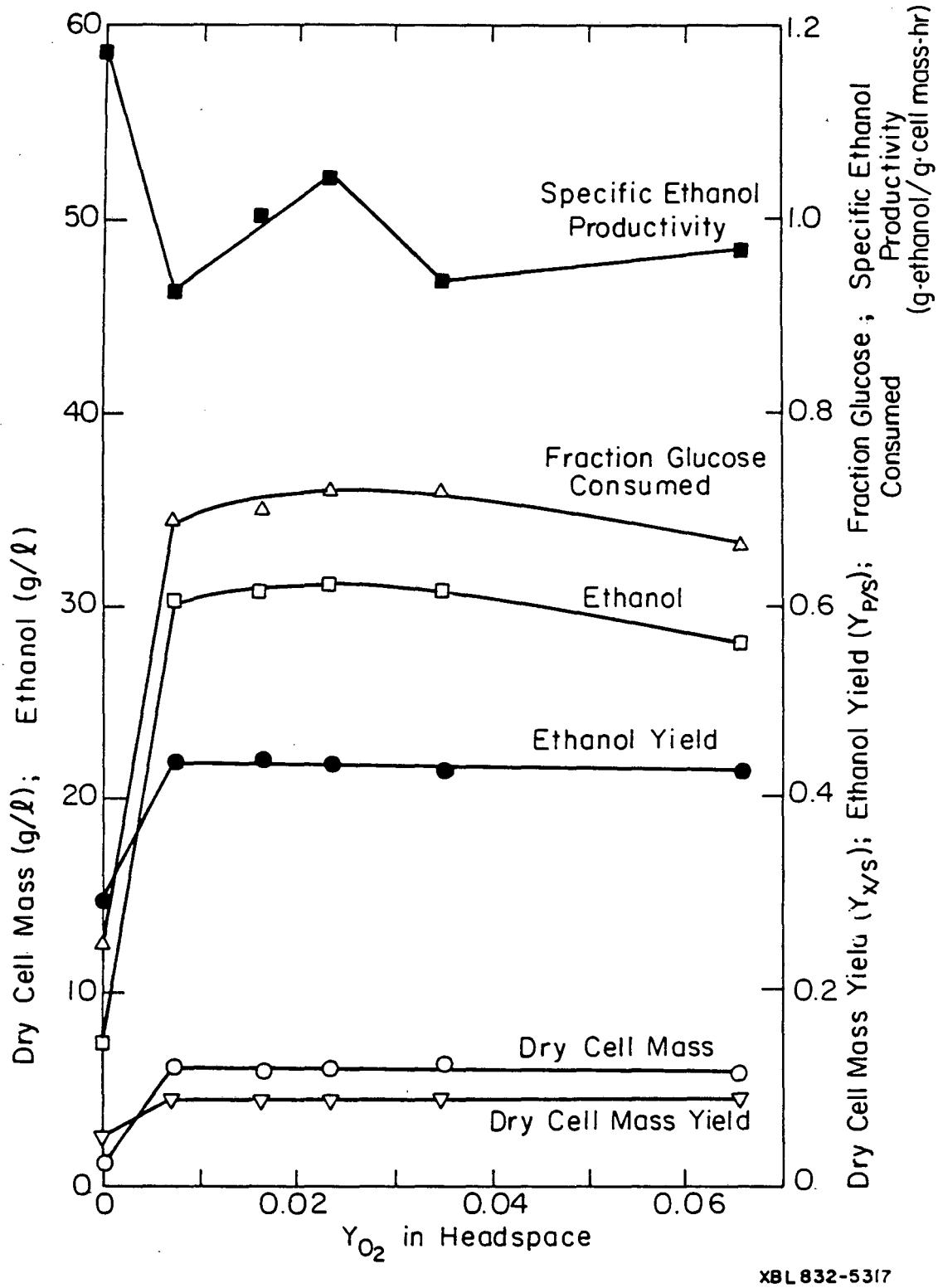

Figure 10.3 Effect of the Oxygen Mole Fraction in the Fermentor Headspace on the Continuous Culture of Saccharomyces cerevisiae, showing Ranges of Minimal Effect, over which Carbon Dioxide Effects can be Studied Separately

and specific growth enzymes in the lipid structure of the membrane. These effects could, furthermore, simultaneously decrease the rate of glucose metabolism. Chen and Gutmanis (6) also found CO_2 to be inhibitory to cell mass yield and respiratory activity for CO_2 partial pressures greater than about 0.3 atm and 0.5 atm, respectively, for aerobic growth of Bakers yeast.

The specific ethanol productivity increase with Y_{CO_2} can be attributed to the decreased cell yield and the slightly increased ethanol yield. The basis for the slight ethanol yield increase is not clear, but it may also be due to diversion of glycolytic intermediates and pyruvate from pathways leading to cell mass components to the pathway for ethanol. However, the ethanol yield increase was marginal. Jones and Greenfield (5) indicated CO_2 did not affect ethanol production.

CO_2 effects on yeast growth and ethanol fermentation are presently not well understood because of complex interactions with the medium and other environmental factors and because of lack of data. Those factors affecting the membrane state, such as ethanol, osmotic pressure, ionic strength and dielectric effects are especially important (5). The need for more studies with yeast is apparent from the presence of only Chen and Gutmanis' (6) aerobic growth data for effects of CO_2 on yeast in Jones and Greenfield's (5) recent review of carbon dioxide effects on yeast growth and fermentation.

10.2.2 Ethanol Effects

Scale-up of 10 g/L glucose feed medium to 100 g/L in continuous cultures resulted in lower percent glucose utilization, cell yield

($Y_{x/s}$) and ethanol yield ($Y_{p/s}$) as discussed in Chapter 7. To determine the extent to which ethanol inhibition was responsible for these effects, continuous cultures were run with the feed medium given in Table 10.1 without and then with 32 g/L ethanol added. This ethanol level approximately represented the additional ethanol concentration developed in fermentation of 100 g/L glucose medium.

Figure 10.4 shows the apparent steady state fermentor cell mass concentration versus dilution rate for continuous cultures with and without ethanol addition. The dilution rate for washout of the original culture without ethanol added was greater than 0.4/hr. Addition of ethanol reduced the washout dilution rate initially to about 0.2/hr. The dilution rate for washout then gradually increased, indicating an increasing maximum specific growth rate and a decreasing ethanol inhibition effect. Adaptation to a higher ethanol concentration in long term continuous culture has apparently raised the ethanol tolerance of the yeast. As seen in Figure 10.5, glucose consumption showed a similar trend in increasing tolerance toward ethanol inhibition as a function of time. The total time for this study was approximately three months.

Because of the continuous adaptation, it was not possible to account for the extent ethanol inhibition reduced cell mass yield and glucose consumption. Direct measurement of ethanol effects could not be accurately obtained because of the relatively high ethanol background concentration from the feed medium. Nevertheless, the ethanol inhibition effects on ethanol production can be approximately followed from the effects on glucose consumption.

Table 10.1

Medium for Study of Ethanol Effects

Component	Concentration
Glucose (g/L)	10.0
$(NH_4)_2SO_4$ (g/L)	0.472
KCl (g/L)	0.0763
H_3PO_4 (g/L)	0.0821
$MgSO_4 \cdot 7H_2O$ (g/L)	0.0495
$CaCl_2 \cdot 2H_2O$ (g/L)	0.0118
Yeast extract (g/L)	0.10
Trace elements (see Table 6.1), (ml/L)	0.5
Biotin (mg/L)	0.004
Ca-pantothenate (mg/L)	1.25
Pyridoxine HCl (mg/L)	1.25
Thiamine HCl (mg/L)	0.133
Penicillin (g/L)	0.050
Ampicillin (g/L)	0.050

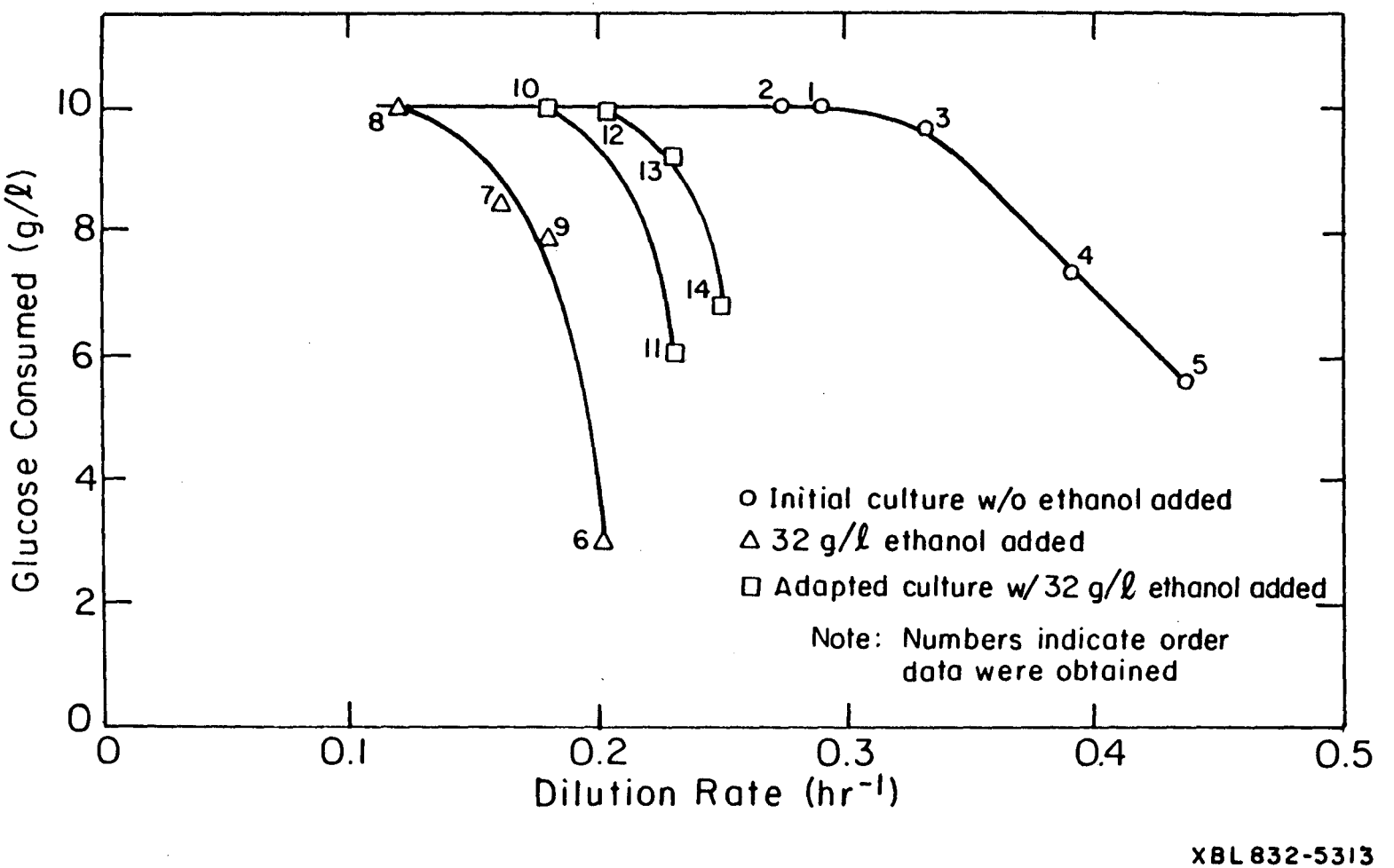

Figure 10.4 Glucose Consumption as a Function of Dilution Rate before and after Adaptation to Ethanol Inhibition in Continuous Culture of Saccharomyces cerevisiae

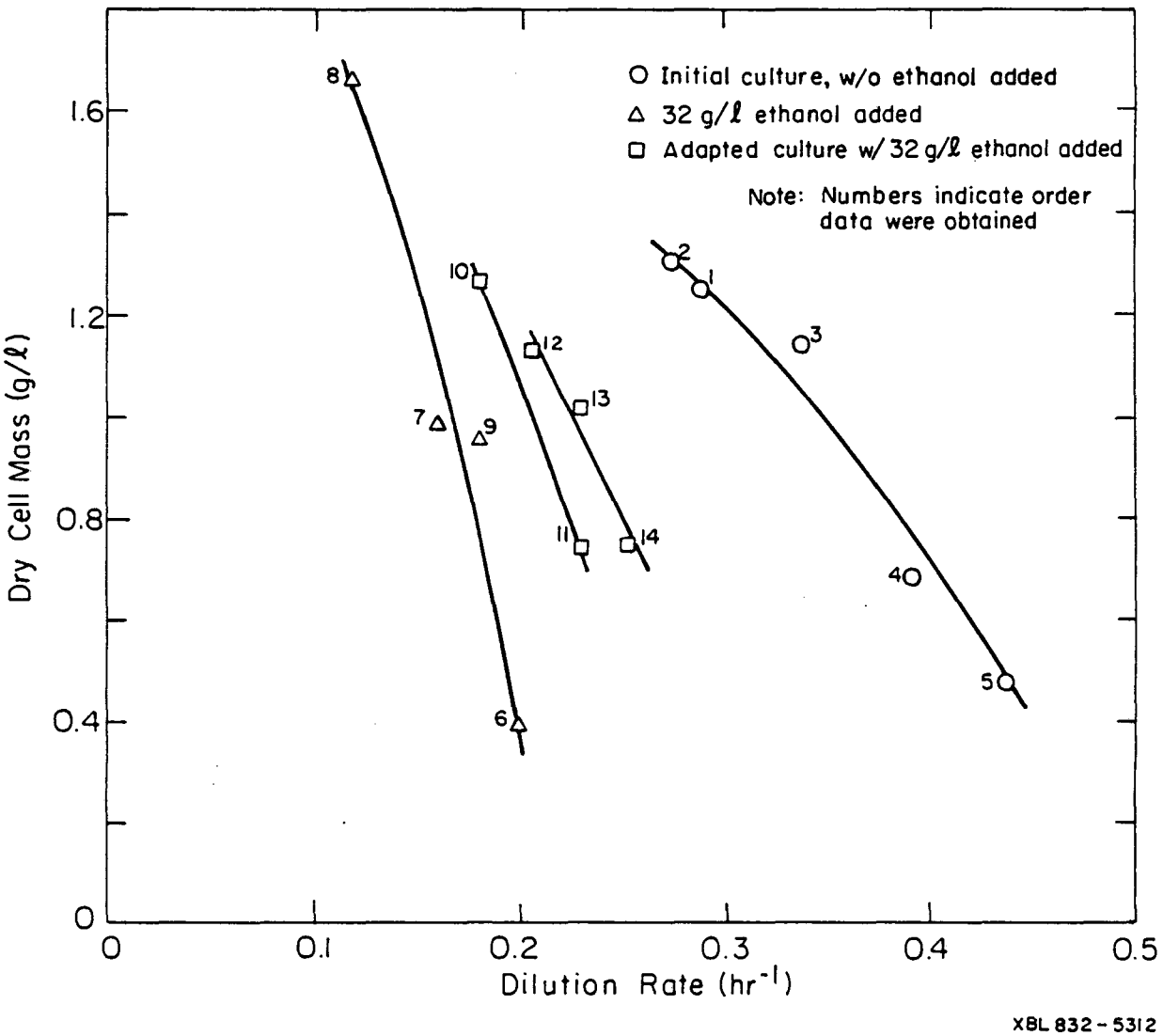

Figure 10.5 Dry Cell Mass Production as a Function of Dilution Rate before and after adaptation to Ethanol Inhibition in Continuous Culture of Saccharomyces cerevisiae

Although Bazua and Wilke (7), Aiba et al (8), Holzberg et al (9), Novak et al (10), Righelato et al (11), Ghose and Tyagi (12), Brown et al (13) and many others have proposed kinetic models for ethanol inhibition effects, they are subject to a number of common limitations and cannot be used quantitatively to predict the ethanol effects for this work. These limitations include neglecting the effects of (1) the medium composition and environmental factors, especially on $Y_{x/s}$ and $Y_{p/s}$, (2) sugar concentration range, and (3) adaptation. The effects of media, temperature, dissolved oxygen and other environmental factors on ethanol inhibition and tolerance are discussed by Day et al (14), Ho (15), and Nagodawithana and Steinkraus (16). Rose and Beavan (17) indicate the effect of ethanol on inhibition of selected glycolytic enzymes (hexokinase and α-glycerophosphate dehydrogenase) and the role of the plasma-membrane lipid composition in ethanol tolerance in Saccharomyces cerevisiae. More fundamental studies considering all these factors are needed for formulating general kinetic models of ethanol inhibition with predictive capabilities.

10.3 Feed Component Inhibition

10.3.1 Glucose Inhibition

During the early stages of fermentations in batch and plug flow reactors, substrate concentrations are close to the feed concentrations. Wang et al (18) indicate that inhibition occurs at glucose levels greater than 100 - 150 g/L with no growth possible for most organisms at 350 - 500 g/L. Since feed glucose concentrations for industrial processes (24) are in the range of partial inhibition, a quantitative measure of this effect is important.

The glucose inhibition effect was measured by determining the initial rates of cell mass and ethanol production in batch cultures with initial glucose concentrations of 100, 200, 300 and 400 g/L. Initial rates were studied to avoid significant ethanol inhibition effects. Beside the glucose, each of the four media contained the following common composition: NH_4Cl, 1.32 g/L; $MgSO_4 \cdot 7H_2O$, 0.11 g/L; $CaCl_2 \cdot 2H_2O$, 0.08 g/L; yeast extract, 8.5 g/L.

As seen in Figures 10.6 and 10.7, the maximum specific growth rate declined significantly with increasing glucose concentration from 100 to 400 g/L. Holzer (19) lists a set of enzymes inhibited by glucose. Wang et al (18) and Pirt (20) suggest dehydration of the cells by the concentrated medium as the mechanism of growth inhibition. Maiorella et al (21) attribute glucose inhibition to osmotic stress. As Maiorella et al (21) noted, the cell mass productivity, based on the maximum specific growth rate of this work, decreased 25% for an increase in glucose concentration from 100 to 270 g/L. A 25% decrease in cell productivity was also noted for the same organism with an increase in glycerol concentration in the feed to 210 g/L in continuous culture. The osmolality of 270 g/L glucose and 210 g/L glycerol solutions are 1.93 and 2.98 Os/kg, respectively (22).

Figures 10.7 and 10.8 show that the maximum specific ethanol production rate decreases slightly from 1.5 to 1.3 g-ethanol/g-cell-hr over the range 200 to 400 g/L initial glucose concentration. This $q_{p\ max}$ decline may be from the association of specific growth and ethanol production rates.

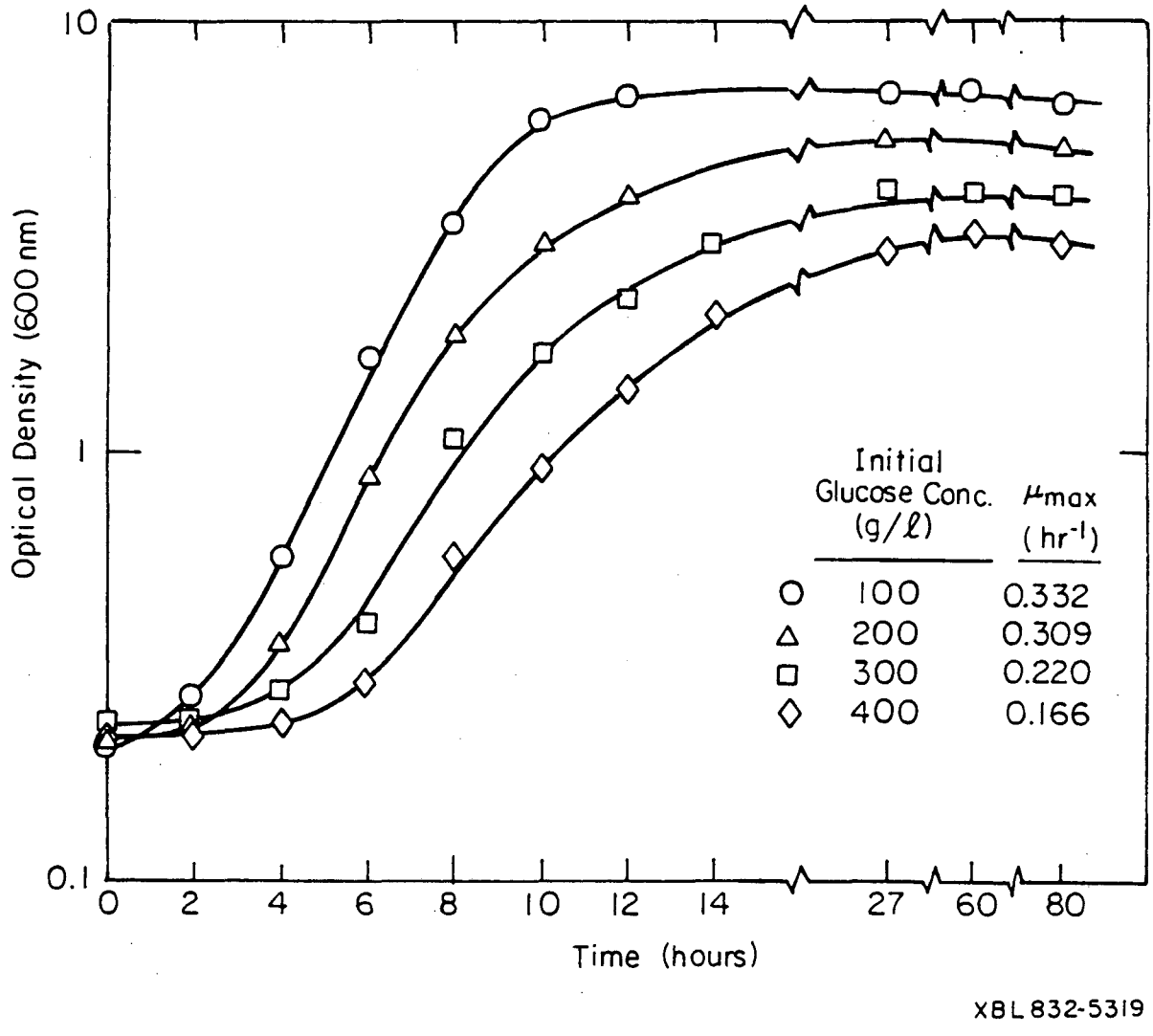

Figure 10.6 Effect of Glucose Inhibition on Maximum Specific Growth Rate of Saccharomyces cerevisiae

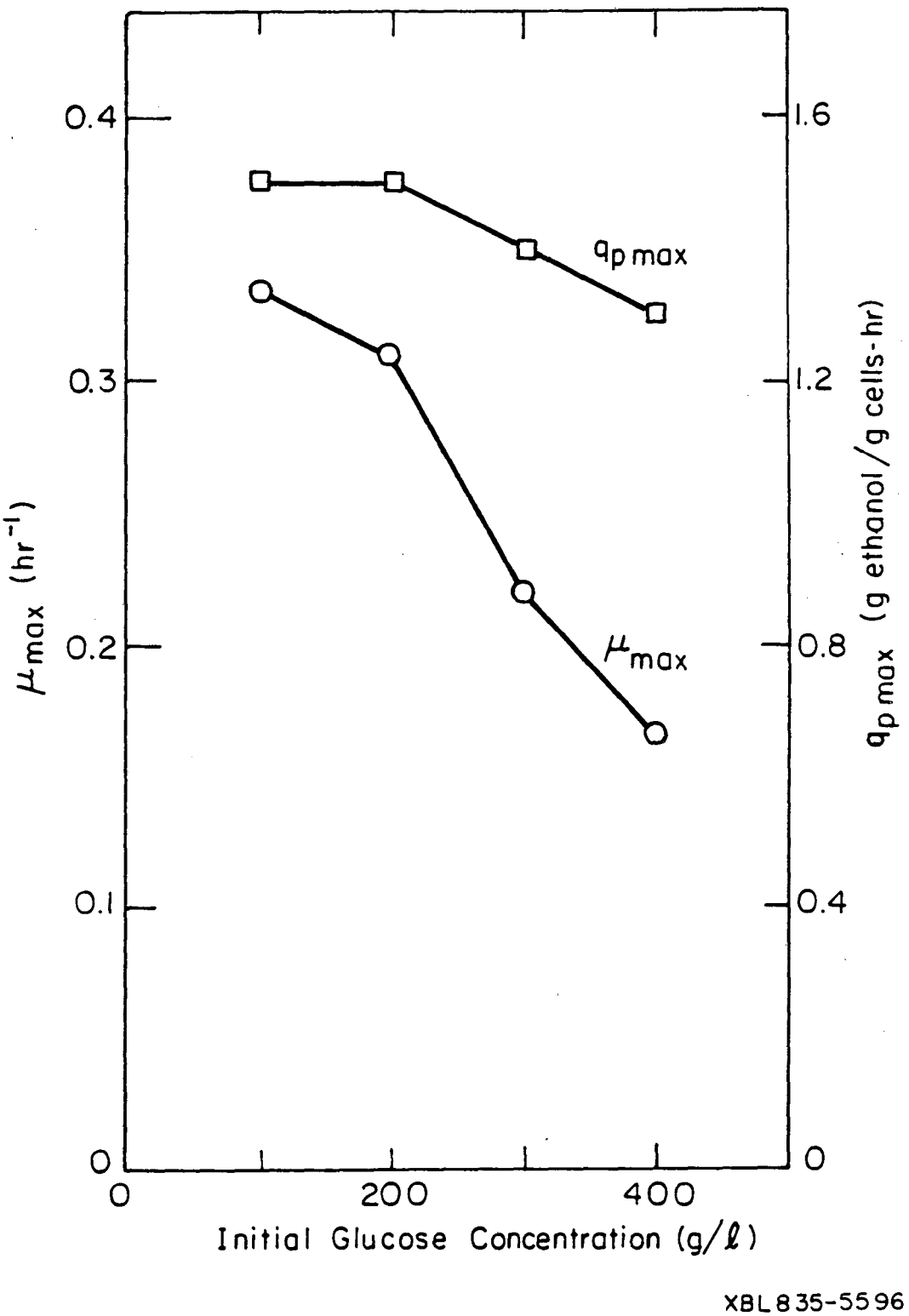

Figure 10.7 Effect of Glucose Concentration on Maximum Specific Growth Rate and Maximum Specific Ethanol Production Rate in Batch Cultures of Saccharomyces cerevisiae (from Figures 10.6 and 10.8)

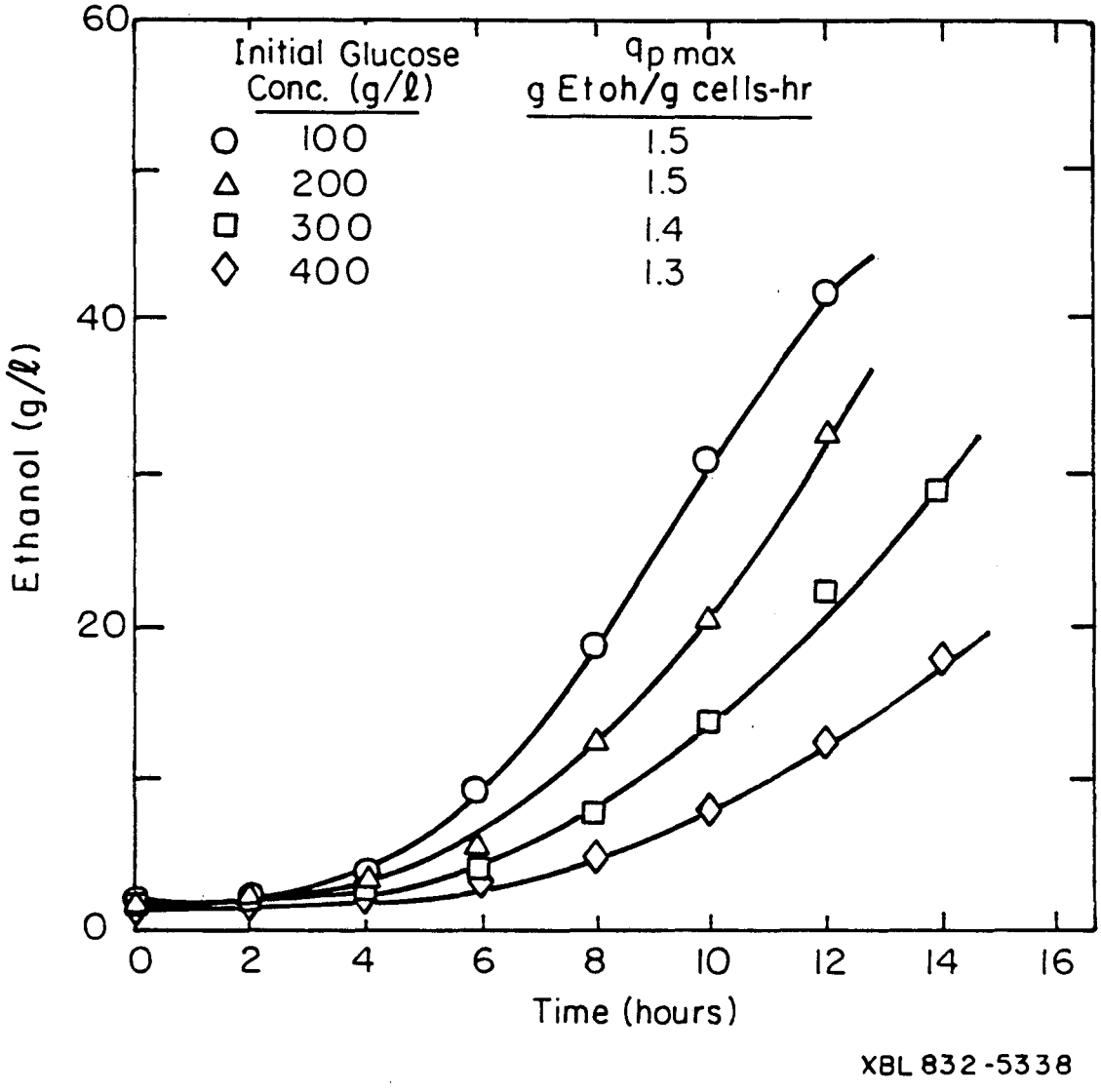

Figure 10.8 Effect of Glucose Inhibition on Maximum Specific Ethanol Production of Saccharomyces cerevisiae

This work is not sufficient to propose a model for glucose inhibition because of the limited data and because of the unknown effect of glucose on catabolite repression. The respiratory quotient would have to be measured (as described in Chapter 9) with varying glucose to determine the effects from varying degrees of respiration and fermentation. Such effects are expected to be minimal, however, because of low dissolved oxygen tension and high glucose concentration in all cases.

10.3.2 Other Feed Components

Excess feed components other than glucose, which is made limiting, are concentrated to high, inhibitory levels in fermentation systems with selective ethanol removal and in stillage recycle systems. These systems are analogous if selective ethanol removal is achieved by vacuum stripping of the fermentation broth in the fermentor or in a separate flash vessel, as described by Maiorella et al (21). The concentration factor can be defined as the ratio of the feed rate to the bleed rate for fermentations with selective ethanol removal (21), and the ratio of the feed rate to the non recycled distillation bottoms rate for continuous stillage recycle systems. High concentration factors in these systems are important because they result in less bleed losses from fermentors with selective ethanol removal and allow more stillage recycle to reduce feed component and waste treatment costs.

Cysewski (1) was only able to obtain a concentration factor of 3.1 at 80% loss in cell productivity in fermentations with simultaneous vacuum stripping of ethanol. He used the medium given in Table 1.1 scaled-up 3.34 fold to maintain the same ratio of feed components with 334 g/L glucose feed. Maiorella et al (21) showed that production rates

of non volatile fermentation by-products for a 334 g/L glucose feed allowed a minimum concentration factor of about 10 with 80% reduction in cell productivity, with acetic acid being the limiting by-product. Maiorella et al (23) also studied the effects of inhibitory concentrations of the major minerals in the feed medium. These minerals should allow a minimum concentration factor of about 6 with 80% reduction in cell productivity, assuming no utilization of Cysewski's (1) explicitly added minerals in his feed for 334 g/L glucose. In this case the limiting major mineral is ammonium chloride at a feed concentration of 3.34 x 1.32 g/L = 4.41 g/L. The major minerals in the feed are not expected to be the limiting inhibitory factors, however, because they should be substantially utilized.

A major component of Cysewski's (1) feed medium not studied by Maiorella et al (23) or others for inhibition is yeast extract. Therefore, yeast extract was considered in this study in search for the limiting factor in concentration of the fermentor broth. Since growth factors are components of yeast extract and of proposed synthetic and semi-synthetic media, their effects at high concentrations were also studied. Cornsteep liquor is considered to be a possible low cost replacement for yeast extract as a source of complex factors; thus, it should also be evaluated for toxic effects at high concentration.

The effects of high concentrations of growth factors, yeast extract and cornsteep liquor were determined by comparing the base media with the concentrated media given in Table 10.2. For inhibition studies of growth factors and cornsteep liquor, their concentrations were increased at least 33.4 fold over their expected feed concentrations to allow for

Table 10.2

Media for Feed Component Inhibition Study

	Base Media		High Concentration Growth Factors Medium (Base #1 + 35 x Growth Factors)	High Concentration Yeast Extract Media		High Concentration Cornsteep Liquor Medium (Base #1 + 35.5 x Cornsteep Liquor)
	#1	#2		#1	#2	
Glucose (g/L)	100.	89.	100.	100.	54.	100.
NH_4Cl (g/L)		1.17		1.32	0.713	
$(NH_4)_2SO_4$ (g/L)	3.54		3.54			3.54
KCl (g/L)	0.625		0.625			0.625
H_3PO_4 (g/L)	0.411		0.411			0.411
$MgSO_4 \cdot 7H_2O$ (g/L)	0.124	0.10	0.124	0.11	0.059	0.124
$CaCl_2 \cdot 2H_2O$ (g/L)	0.0295	0.0708	0.0295	0.0795	0.0429	0.0295
Trace elements (see Table 6.1), (ml/L)	2.5		2.5			2.5
Biotin (mg/L)	0.00527		0.189			0.00527
Ca-pantothenate (mg/L)	2.0		72.1			2.0
Thiamine HCl (mg/L)	1.34		48.3			1.34
Pyridorine HCl (mg/L)	1.0		36.1			1.0
Myo-inositol (mg/L)	1.25		45.4			1.25
Cornsteep Liquor (g/L)	2.0		2.0			71.
Yeast Extract (g/L)		7.56		88.	156.	
Penicillin (g/L)	0.30		0.30			0.30
Ampicillin (g/L)	0.0375		0.0375			0.0375
Streptomycin (g/L)	0.01		0.01			0.01

the use of 334 g/L instead of 100 g/L glucose feed and to allow a minimum concentration factor of 10 before the component becomes the limiting factor.

Table 10.3 presents the results of the comparisons. A 36 fold increase in the growth factors improved sugar utilization to practical completion with the same cell mass and ethanol yields. Hence, concentration of growth factors to this level provides a positive effect.

Cornsteep liquor at 71 g/L improved glucose utilization, but maintained about the same cell mass and ethanol yields. Thus, from an inhibition standpoint, cornsteep liquor is suitable as a source of complex factors.

The yeast extract concentration of 88. g/L represents Cysewski's (1) feed concentration times the concentration factor which caused 80% decrease in cell productivity, i.e., 3.34 x 8.5 g/L x 3.1. However, this concentration of yeast extract stimulates rather than inhibits cell mass and ethanol productivity with respect to Base Medium #1. These productivities from 88. g/L yeast extract are, in fact, very close to those found by Cysewski (1) for Base Medium #2. Increasing the yeast extract concentration to 156 g/L also showed no adverse effects as measured by glucose utilization and cell and ethanol yields. To determine if an inhibitory product was formed from the yeast extract during autoclaving, the 88. g/L yeast extract with minerals mixture was then autoclaved at 135 ^{o}C. for about 4 hours instead of at 124 ^{o}C. for 1 hour. (The glucose was sterilized separately from yeast extract and minerals in all cases in this study and in Cysewski's studies (1) with 334 g/L glucose media). Autoclaving the yeast extract at extreme conditions did

Table 10.3

Effect of High Concentrations of Selected Feed Components

Medium (see Table 10.2)	Dilution Rate (1/hr)	Dry Cell Mass Conc. (g/L)	Ethanol Conc. (g/L)	Glucose Conc. (g/L)	q_p (g-etoh/ g-cell-hr)	$Y_{p/s}$	$Y_{x/s}$
Base Medium #1	0.168	8.32	36.5	7.3	0.74	0.39	0.090
High Conc. Growth Factors Medium (Base Medium #1 + 35 x Growth Factors)	0.168	8.96	37.1	0.5	0.70	0.37	0.090
Base Medium #1	0.266	4.88	24.0	39.9	1.3	0.40	0.081
Base Medium #2 (from Cysewski(1))	0.27	9.7	32.5	15.	0.90	0.43	0.13
High Conc. Yeast Extract Medium #1 (100 g/L glucose, 88 g/L yeast extract)	0.266	10.1	33.5	13.9	0.88	0.39	0.12
High Conc. Yeast Extract Medium #2 (54 g/L glucose, 156 g/L yeast extract)	0.266	6.52	20.5	1.7	0.84	0.39	0.12
High Conc. Yeast Extract Medium #1 autoclaved at 135 ^{o}C. for 4 hr	0.261	8.83	33.0	24.2	0.97	0.43	0.12
Base Medium #1	0.20	6.02	32.9	23.4	1.1	0.43	0.079
High Conc. Cornsteep Liquor Medium (Base Medium #1 + 71 g/L Cornsteep Liq.)	0.20	7.70	42.0	4.9	1.1	0.44	0.081

decrease glucose utilization from 86 to 76%, but did not affect the cell yield and increased the ethanol yield. The inhibitory factor limiting Cysewski (1) to a concentration factor of 3.1, therefore, does not appear to be from the yeast extract.

A possible explanation for Cysewski's inhibition is a synergistic effect from the inhibition of ethanol, CO_2, secondary by-products and unutilized feed components. However, much more research is needed to test all these possibilities. Additional work is also required to determine the actual distribution and concentrations of secondary by-products for the conditions employed by Cysewski (1). Maiorella et al's (21).concentration factors for inhibition assume the by-product concentrations reported by Neish and Blackwood (25). By-products production rates, however, are affected by the medium and environmental factors.

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Chapter 11

11. Conclusions and Recommendations for Future Work

In addition to specific conclusions given at the end of previous chapters, some general overall conclusions and recommendations for future work can now be made.

Media requirements among yeast, even among strains of the same species, differ significantly. Because of the diverse biosynthetic capabilities for growth factors, differences in these requirements are especially great and must be determined experimentally for a given strain. Continuous cultures are more effective than batch cultures in determining media requirements because inoculum effects can be eliminated. The method of pulse injections of nutrients combined with shifts in feed media concentrations was useful in establishing requirements for media components. Medium composition could also be approximately optimized with this method, but multi-variable changes made separating the effects of individual medium components difficult. Systematic variation of feed component concentrations one at a time allowed formulation of media optimum with respect to the major fermentation parameters. Furthermore, a conceptual model was proposed to relate the effects of the major inorganic nutrients, growth factors and complex factors to structural and catalytic functions in the cell. This conceptual model provides a general basis for understanding and predicting the trends of various fermentation parameters as a function of medium composition.

The conceptual model for nutrient effects can be improved with more data for all feed components, especially phosphates, magnesium, calcium, and trace elements. The effect of varying a single nutrient with different base media should be studied to determine the extent of interaction effects. Media optimized with respect to the major fermentation parameters, as given in Tables 7.10 and 7.11, should also be experimentally tested. Furthermore, measurement of the fermentor effluent medium and cell compositions for each nutrient studied will provide mass balances and allow formulation of kinetic models for nutrient uptake. The effect of dilution rate should also be determined for nutrients which vary in intracellular concentration with specific growth rate.

Another major limitation to specific media requirement studies is the variation in requirements for a given strain with adaptation. Through long term continuous culture in minimal medium, the yeast strain studied appeared to have reduced or lost requirements for a number of growth factors. This yeast was eventually capable of significant growth and an ethanol productivity of 5.6 g/L-hr with a completely synthetic medium in continuous culture. The original yeast required significant yeast extract for the same level of ethanol production. Further studies of the stability and reversibility of the adaptation changes are important. Koser (1) reports similar cases of induction and repression of enzyme activity and of possible mutational changes. Therefore, media requirements for a process need to be determined with the actual organism, raw materials, and environmental conditions to be used. Many of the trends observed in this study should, nevertheless, be applicable for organisms with similar metabolism.

The non-nutrient role of feed components in complex feedstocks should also be investigated. Jones et al (2) claim that synthetic media studies cannot be applied to industrial fermentation media, which have natural chelating agents, such as amino acids, to inactivate heavy metal ions and other inhibitors. This claim implies synthetic media have more stringent conditions for fermentation, but also that improvements can be made by studying the effect of various chelating or complexing agents for suspected inhibitors.

More complete and accurate analyses of raw materials are important to determine the level of possible inhibitors and growth factors in industrial fermentations to allow better simulation with synthetic media and to determine the extent of nutrient assimilability. For example, White's (3) claim that half of the phosphates in molasses are unassimilable and Solomons' (4) claim that beet molasses have substantial unusable nitrogen need to be checked. These analyses would also have a major effect on the economic optimization of media formulation as reported in Chapter 8.

The commercially available dissolved oxygen probes at present do not appear capable of measuring dissolved oxygen in fermentation broths in the 1 - 10 parts per billion range, the region of interest in ethanol fermentation according to Cysewski (5) and Nishizawa et al (6). Nevertheless, the trends of the major fermentation parameters could be observed in this range. Approximate oxygen uptake rates under fermentative conditions could be calculated and compared with the requirements for lipid biosynthesis with an assumed lipid composition. This composition of lipid and other possible fermentative requirements for oxygen

should be determined in future work. The observed oxygen consumption rates support a passive diffusion mechanism for oxygen transport into the cell with negligible diffusional resistances; therefore, the internal cell reaction rate for oxygen controls the O_2 uptake rate. Measurements of the diffusion coefficient of O_2 through the cell wall and cell membrane are needed to provide a more accurate O_2 profile from the medium into the cell.

Media requirements are affected by the environmental conditions. For example, synthetic growth factors could substitute for yeast extract more effectively in continuous cultures with low glucose feed concentrations and, thus, low ethanol concentrations. Environmental inhibition effects, such as from ethanol, CO_2 and excess substrates, need to be better understood and separated from media deficiencies and other environmental effects. The ethanol inhibition effect is particularly important, but also complex, because of adaptation to ethanol and because of the different inhibitory effects of intracellularly produced and externally added ethanol (7,8). More work is required also in isolating carbon dioxide effects from oxygen and other environmental factors.

In conclusion, an improved understanding of the nutrient requirements and environmental factors in ethanol fermentation has been gained and should increase productivity, allow more efficient use of feedstocks, and thereby lower the cost of ethanol. Much additional research, nevertheless, is still required.

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