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Post-harvest open field burning of straw has been a common practice of waste disposal and field sanitation for the grass seed industries in the Willamette Valley for over 20 years. This practice has resulted in excessive air pollution. Acceptable new techniques are needed for disposal and or utilization of straw.

One possibility would be the industrial production of food and fodder yeast protein from grass straw. Continuous aerobic propagation of yeast on assimilable sugars present in straw hydrolyzates may be a feasible alternative to burning.

Milled Annual Ryegrass (Lolium multiflorum) straw (20-40 mesh) was used in this study. The straw was hydrolyzed in an autoclave with 3% sulfuric acid for 30-45 minutes. The hydrolyzate obtained was fortified with NH_4OH as a nitrogen source. The pH was adjusted to 4.0 and the fortification completed with the addition

of KCl, H_3PO_4 and magnesium sulfate. After filter sterilization, the fortified hydrolyzate was used as a medium for continuous aerobic propagation of Candida utilis (strain NRRL Y-1084) at 32°C. The effect of sugar concentration and yield constants were studied. The yield constants were found to be in the range of 0.4 to 0.63. Gas-liquid chromatographic analysis of sugars in the effluent media indicated that C. utilis preferentially utilized hexose to the pentose sugars. Maximum total sugar utilization was 82%. Arabinose which contributed10 - 15% of the total hydrolyzate sugar was not assimilated.

Lyophyilized yeast cells were off-white in color, odorless and contained about 48 - 53% protein on dry weight basis as determined by Kjeldahl nitrogen analysis.

Data obtained in this preliminary investigation have shown that Annual Ryegrass straw hydrolyzate can serve as a good medium for Candida utilis production.

Production of *Candida utilis* from Annual Ryegrass Straw Hydrolyzate

by

Chayavoot Lekprayoon

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PRODUCTION OF CANDIDA UTILIS FROM ANNUAL RYEGRASS STRAW HYDROLYZATE

INTRODUCTION

Open field burning of the waste straw has been the method of disposal and field sanitation for the small grain and grass seed industry in the Willamette Valley for many years. It is estimated that between 1 and 1.5 million tons of straw are burned annually in this area. This practice causes excessive air pollution which is intolerable. The ready availability of such a large amount of waste straw which is now burned has prompted investigators on this campus to study methods of utilizing this material for conversion into useful products.

Because of the rapidly increasing world population and consequent increase in food demand, especially in the under-developed countries, a considerable amount of research has been directed toward finding cheap and alternate sources of food, especially protein. The major source of human food protein in the world is cereal. Annually, out of about 82 million tons of protein presently available, over 40 million tons are supplied by cereal with approximately 25 million tons derived from animal products (3). Fortification of cereals with food supplements to obtain necessary amino acid content and balance for normal growth and development appears to be

the simplest and quickest way to reduce current acuteness of the food problem.

Because straw, a waste material from agriculture is readily available and low in cost, development of an economic process and technology which could convert this available waste into usable food protein deserves serious consideration.

This study was undertaken to investigate the proper conditions necessary to attain optimum growth of Candida utilis in a continuous fermentation system using ryegrass straw hydrolyzate as substrate.

The gross composition of ryegrass straw consists of cellulose, hemicellulose, lignin and ash. If the polymeric sugars in cellulose and hemicellulose could be released by a combination of physico-chemical treatments, the treated material could be used for the production of single-cell protein. Preliminary studies in the Department of Microbiology at Oregon State University revealed that the hydrolysis of straw with dilute sulfuric acid at elevated temperature yielded a hydrolyzate in which Candida utilis, a high protein yeast, could be successfully propagated with very little fortification.

LITERATURE REVIEW

The production of yeast protein had attained commercial-scale propagation by the beginning of World War II. Critical wartime shortages of food stimulated widespread interest and a rapid development of the technology to utilize cheap sources of carbohydrates for the biosynthesis of protein (1, 18, 26). Besides Saccharomyces spp., Candida utilis is very important in the manufacture of food yeast. Interest in utilizing C. utilis for feeding purposes originated in 1940 when Lechner (27) reported the pentose-utilizing abilities of this yeast.

The earlier nomenclature for C. utilis was Torulopsis utilis (or Torula Dried Yeast by trade name). Later, Lodder and Kreger-van Rij (30) placed this organism in the genus Candida. They described C. utilis as a nonascospore-forming yeast, ovoid to cylindrical in shape measuring 7 microns in length and 4 microns in width. They characteristically produced pseudomycelia under anaerobic conditions, and were capable of assimilating glucose, and some of xylose, but lacked the ability to utilize arabinose.

Germany was the first country to give serious attention to the large scale propagation of C. utilis as a food supplement (43), exploiting their ability to attack a wide range of carbon and nitrogen compounds and to grow in the absence of accessory growth factors

such as biotin (6). C. utilis also grows well at low pH (18, 40) so that bacterial contamination of acidic fermentative liquors is not a serious problem. Further advantages of using C. utilis include their high protein content (19), and adaptibility to large scale industrial fermentations utilizing spent sulphite liquor, wood hydrolyzate and modified molasses as substrate.

The onset of World War II with resultant dearth of food for civilian populations also stimulated interest in yeast process development in many countries throughout the world.

In England, an experimental scale fermentation was started by Chemical Research Laboratory in Teddington. This early process has been extensively reviewed by Floro, Williams, Flook and Collier (16). Ammonium sulphate and phosphate were added to crude cane molasses as sources of nitrogen and phosphate. Fermentation was carried out at 30 C with vigorous aeration and a pH around 4.0-4.5. The highest yield attained in these trials amounted to 50% dry weight yeast based on sugar used. Subsequently, improved strains of this species were developed by the treatment of the yeast with camphor. One of these, C. utilis var. thermophilia is suitable for high temperature propagation. Another strain was characterized by unusually large sized cells, which were relatively easy to harvest by centrifugation.

In the U.S.A., development of yeast technology followed a

different trend from those of Germany and England. Most of the investigations in the U.S.A. were directed toward utilizing the waste carbohydrate substrates, such as fruit and vegetable trimmings (47), paper industry wastes (18), and agricultural wastes such as corncobs, oat hulls and bagasse (37) for yeast propagation to be used mainly as supplements in animal feeding.

In addition to the food yeast manufacturing plants built during the war in Germany, England and United States, Taiwan's recent industry engaged in producing yeast tablets for human consumption from cells grown on black strap molasses was brought to focus by Chien (10). Three grades of dried Torula yeast were produced. Taiwan yeast tablets, with protein content of not less than 45 percent and thiamine content up to 90 micrograms per 100 grams yeast, were sold as superior grade.

While yeast technology has developed in many countries over a period covering 30 years or more, the first production of yeast protein from woodwaste was reported from Bulgaria in 1966 (35). In the same year, Mexico started experimental scale production of yeasts from the same source (44).

Propagation Technology

The production parameters involve treatment of raw materials to be used as the substrate for yeast growth, control of temperature,

pH and aeration.

Raw Materials

As mentioned earlier, C. utilis is one of the most versatile yeasts known and can attack a wide range of carbon sources, and a variety of raw materials including waste fruit juices and molasses, and hydrolyzates of agricultural wastes. The techniques and details of food and fodder yeast production are extensively reviewed by Lipinsky and Litchfield (28), Peppler (37), and Snyder (45). Straw is also known as a good carbohydrate source, but the information available on straw-derived carbohydrates for use as a microbial substrate is limited. Most of the literature is in Russian with English summary (11, 12, 48) and is neither inclusive nor entirely complete for immediate application.

In general, the use of any of these raw materials for yeast propagation is always attended by the following considerations:

1) Pretreatment of the raw material to produce the maximum quantity of assimilable sugar and other carbon containing materials for the organisms under investigation.

2) Determine the mixture with respect to sugar concentration to produce a good nutrient medium.

3) Determination of the necessary quantities of inorganic salts to be added to achieve maximum growth.

In addition to carbon source, yeasts require nitrogen, phosphorus, potassium and other trace elements. The amount of inorganic supplements required for yeast production is quite dependent on the particular substrate used.

Aeration

The amount of air supplied to the fermentor and the manner of its dispersion are known to be critical factors in yeast production efficiency. The peculiar manifestation of yeast metabolism, also called Pasteur effect, is the great increase in growth which occurs when the medium in which the yeast is growing is well aerated. The importance of aeration has been the subject of a number of excellent studies (4, 46) and reviews (14, 45). Inadequate aeration in yeast propagation results in the production of alcohol rather than cell substance, which is reflected as a loss in cell yield. The lower the aeration rate, the greater is the percentage of metabolic carbon dioxide derived from the decarboxylation of pyruvate via glycolysis, while that derived from the oxidative tricarboxylic acid pathway rapidly declines which results in the loss of energy and lower cell yields (8). Additionally, the synthesis of nucleic acid is more rapid in anaerobically grown cells than those grown aerobically (50). This has a direct bearing on the suitability of yeast as a source of single-cell protein for human consumption. If yeasts make up a

large portion of a human food intake, the large nucleic acid content would cause an increase in uric acid levels in the blood stream (33) which can lead to toxemia. Overaeration, however, serves no useful purpose and results in sizable added expenses of air compressor and antifoam system operation.

The importance of maintaining proper aeration for efficient yeast propagation led to the development of devices for measuring the residual oxygen in the fermentation system (46). Recently, Johnson et al. (25) developed a simple and convenient steam-sterilizable membrane probe for continuous monitoring of the dissolved oxygen level in fermentors. The probe can withstand repeated steam sterilizations, and consists of a silver cathode covered with teflon membrane, a lead anode housed in a glass tube containing acetate buffer as an electrolyte.

Since, the efficiency of aeration varies widely with different substrates and with different types and sizes of fermentors, a single, universal, generalized specification of air requirements cannot be made. Finn (15) stated that less total air is required if the fermentor is made tall. This is because the air is being scrubbed more thoroughly in its passage through the mash.

Agitation

Another important parameter affecting the efficiency of aerobic

fermentation is agitation. Agitation chops up the air stream into small bubbles, thus increasing interfacial area, and minimizing the thickness of liquid film around individual air bubbles. It also retards the normal escape rate of bubbles by circulating the liquid in a swirling motion.

Better aeration and improved gas absorption can be attained by adjusting agitation.

pH

The control of pH is important from a number of stand points. The cell yield, contamination control, foaming of the medium and the color of the yeast are all dependent upon changes in pH of media during fermentation. The pH selected will depend upon the raw material used and other factors. Normally, industrial plants producing C. utilis operate in the pH range of 4.0 to 6.0. Peppler (36, 37) reported a pH range of 4.0 to 5.0 for growing yeast in cane molasses, while Peterson (40) employed pH values between 4.5 to 5.5 in a wood hydrolyzate system. Walker et al. (51) operated at a pH range of 5.5 to 6.0 for growing C. utilis in spent sulphite liquor.

The pH of operation may be controlled according to accepted practices. Automatic pH controller system is commonly employed. Ammonia, lime, sulfuric acid and other agents are widely used in

automatic systems for addition to the fermentor in proper amounts when desired.

Temperature

The maintenance of an even temperature in the fermentor is recognized to be a prime factor in efficient yeast growth in the continuous phase. Any microorganism will grow rapidly as the temperature is increased, but the limits of temperature tolerance vary. For normal growth of C. utilis, temperature should be maintained in the range of 30-32 C (23, 29). Lowering the growth temperature from the optimum was shown by Brown and Rose (5) to increase the ribonucleic acid content and the cell volume of C. utilis grown under NH_4^+ limitation. Besides the low yield, the high ribonucleic acid content of the cell also is not desirable in food yeast.

In geographical locations where the diurnal temperature is normally elevated and where refrigeration is not practical, C. utilis var. thermophlia, which has a high optimum growth temperature, may be used.

Nutritive Values of C. utilis

The production of C. utilis was originally developed so that the protein and amino acid values of foods and feeds in periods of normal food and feed shortage could be increased by yeast supplement.

Presently, C. utilis is used mainly as a food supplement because of its high qualitative and quantitative vitamin content rather than protein content. Comprehensive details on protein and vitamin content of this organism can be found in many recent reviews (33, 37, 45). According to Inskeep et al. (23) and Peppler (38), C. utilis is rich in isoleucine, lysine and other amino acids in comparison with egg protein which is the reference protein recommended by the joint FAO/WHO expert group, but is relatively deficient in the sulfur containing amino acids such as methionine and cystine. Attempts to increase the sulfur amino acid content of C. utilis protein by the addition of choline, threonine and cystine to the media were unsuccessful (9).

Wiley (53) demonstrated that, in addition to being rich in protein, C. utilis is also high in vitamins, particularly, the vitamin B complex which is important for human and non-ruminant animal nutrition. He also stated that vitamin D_2 may be obtained by irradiating the yeast with ultraviolet light, thus transforming ergosterol to calciferol.

Although, C. utilis is rather high in nutritional value in terms of protein and vitamin content, many people are still cautious in its use as food because of toxicity observed in long term feeding experiments. Thus far, there are no indisputable and conclusive reports to prove the toxicity of this organism to humans. The work of

Edozoien et al. (13) demonstrated that the lowest level of yeast nucleic acid fed, about 2.9 grams per day, increased the uric acid excretion in young adults to more than 800 micrograms per day, and any additional increment was proportional to increase in nucleic acid ingestion. Maul (32) attempted to reduce the nucleic acid content in yeast cells by heat shocking for a few seconds at 45-70 C followed by short incubation at 45-50 C and 55-60 C; 80 - 85% reduction of nucleic acid content in the yeast cells was reported.

MATERIALS AND METHODS

Raw Material

A single source of Annual Ryegrass (Lolium multiflorum) straw grown in the Willamette Valley, was used throughout this study. Straw was milled to an average mesh size of 20-40 and was supplied by the Department of Farm Crops at Oregon State University. The elemental analysis of straw (Table 1) was determined by the Department of Agricultural Chemistry, Oregon State University, Corvallis Oregon. The percentage is based on the dry weight of straw. Moisture content of the straw ranged from 9-10%.

Straw Hydrolysis and Media Preparation

Exactly 1200 grams of milled straw were suspended in 12 liters of 3% (w/v) technical grade sulfuric acid (Van Waters and Rogers, specific gravity 1.835). Before hydrolysis, the straw was moistened with the acid solution by stirring the slurry. To determine the optimum straw hydrolysis time in terms of the greatest sugar release, suspensions of straw in 12 liters of 3% sulfuric acid were hydrolyzed in an autoclave for 15, 30, 45 and 60 minutes. The hydrolysates from each of these batches were analyzed for individual pentose and hexose sugars by the method which will be described later.

Table 1. Analysis of Annual Ryegrass straw.

Component	% of dry weight	Elemental Composition, % Dry Weight								
		C	H	O	N	Na	Ca	K	P	Other
Cellulose	46.0	20.5	2.9	22.6	0.9					
Lignins	16.0	11.8	1.1	3.1						
Pentosans	25.0	11.4	1.5	12.1						
Protein	6.0	2.4	0.5	2.2	0.9					
Fats	1.0	0.8	0.1	0.2						
Ash	6.0					0.2	0.2	1.0	0.2	4.4
	100.0	46.9	6.1	40.1	0.9	0.2	0.2	1.0	0.2	4.4

The hydrolyzate used as the medium for the growth of C. utilis in this investigation was prepared as outlined in Figure 1. The 1200 grams of straw in 3% sulfuric acid suspension were hydrolyzed by heating in an autoclave for 30-45 minutes at 121 C, 15 psi steam pressure. The straw residue was separated from the liquid hydrolyzate by straining and squeezing the residue with a wine press. The residue was washed with 4 liters of water and pooled with the rest of the hydrolyzate. Ammonium hydroxide, 4.2 ml. per liter of straw hydrolyzate, was added as a nitrogen source and the pH of the solution was then adjusted to 4.0 by the addition of calcium oxide. The neutralization product, calcium sulfate, was allowed to settle out and the supernatant hydrolyzate was siphoned off. The calcium sulfate sludge was washed with 2 liters of water and the wash water was added to the hydrolyzate. The sludge was then discarded. Fortification of the hydrolyzate for yeast growth was completed by additions of 0.25 ml. phosphoric acid, 0.44 gram potassium chloride and 0.1 gram of magnesium sulfate per liter of hydrolyzate. These N, P, K and Mg levels would theoretically support a yeast population of 10 grams dry weight cells per liter in the presence of adequate oxygen and carbon sources (20). Since experimental yields were always less than 10 gram cells per liter, these inorganics were never limiting. The fortified hydrolyzate was further clarified by passing through an Ertel Filtration Unit (Ertel Engineering

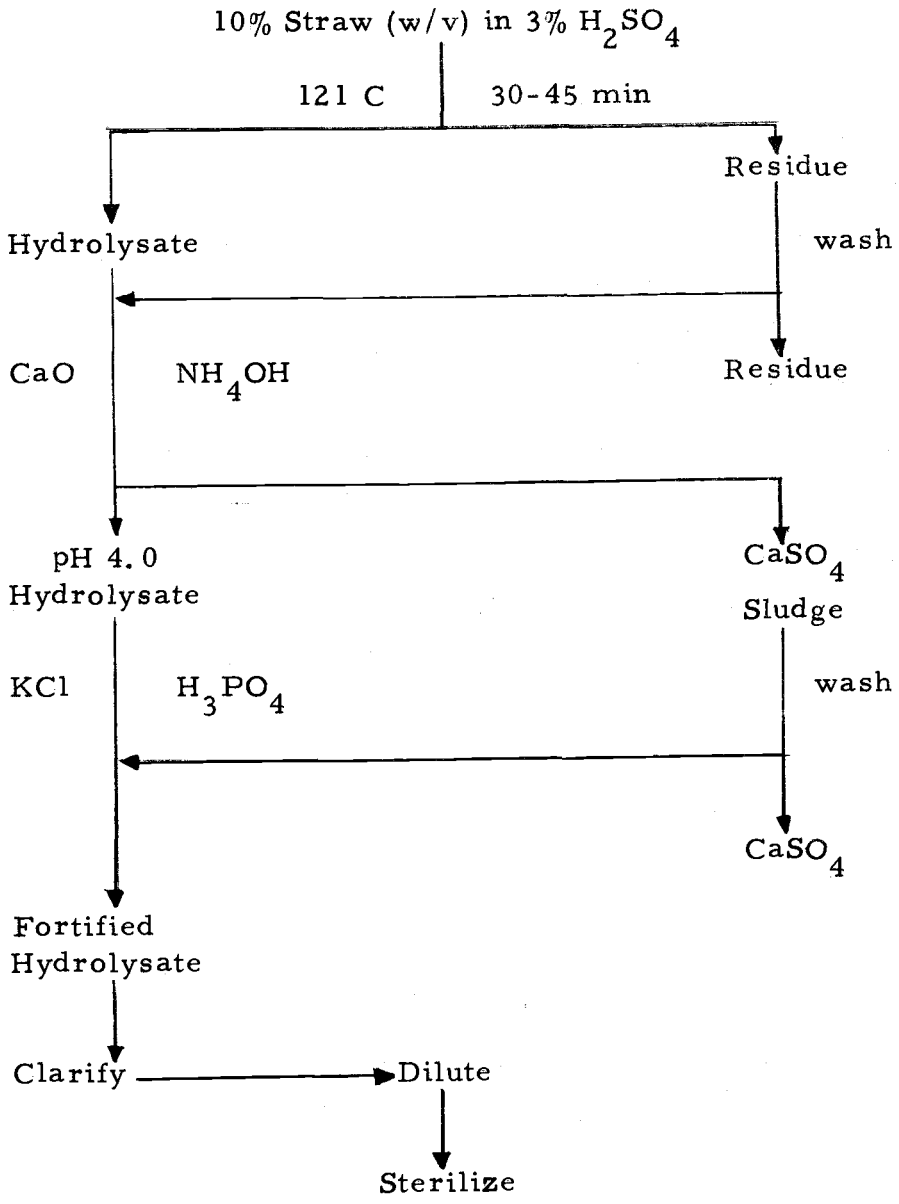


Figure 1. Hydrolysis of straw and medium preparation.

Corporation, Kingston, New York) using a combination of no. 5 and 7 filter pads. After clarifying, the same unit, equipped with no. 7 and 9 filter pads, was used to filter sterilize the foritified hydrolyzate.

Sugar Analysis

Gas-Liquid Chromatography

Qualitative and quantitative sugar analyses of both influent and effluent media were made by gas-liquid chromatography. The gas chromatographic analyses were made with a F and M Scientific Corporation gas chromatograph, Model 402 (F and M Scientific Corporation., Avondale, Pennsylvania), equipped with a dual column and dual hydrogen flame ionization detector system. Chromatographic peak area measurements were made with an electronic integrator, model 3370 A, manufactured by Hewlett-Packard, Avondale, Pennsylvania.

Preparation of Column

One hundred milligrams of ethylene glycol succinate (F and M Scientific Corporation) were dissolved in 25 ml. of chloroform and 100 milligrams ethylene glycol adipate (Applied Science, State College, Pennsylvania) were dissolved in acetone. The above

mixtures were quickly poured together, mixed with 10 grams of Gas Chrom P, 100-120 mesh (Applied Science), and occasionally stirred, gently. After 30 minutes, the mixture was allowed to drain off and residue dried at room temperature in a large petri dish. About 6 ml. of this powder was packed, with vibration into a 4 ft. x 1/8 inch o.d. stainless steel column. Both ends of column were plugged with glass wool and a newly prepared column was conditioned overnight at 180 C by purging with the carrier gas (helium).

The column was operated isothermally at 180 C with an injection port temperature of 215 C and a detector temperature of 210 C. The approximate gas flow rates were as follows: helium 70 ml./min., hydrogen 40 ml./min., and air 230 ml./min.

Preparation of Alditol-Acetate Derivatives

Sugars in the samples were converted into their alditol-acetate derivatives by a modification of the procedure reported by Albersheim et al. (2). To 10.0 ml. of straw hydrolyzate approximately 1.5 grams of barium chloride was added to precipitate the sulfate group which is an interferring substance. The precipitate was removed by centrifugation and the clarified sample was adjusted to pH 6.0 by the addition of barium hydroxide. Exactly 2.5 ml. of the fluid was transferred into 250 ml. round bottom flask and 1.0 ml. of a myo-inositol solution containing 10 milligrams of the compound was added as an

internal standard. The sugars in the liquid were then reduced to their repective alcohols by the addition of 0.15 grams of sodium borohydride. After 30 minutes of reaction time at room temperature, the excess sodium borohydride was decomposed by the dropwise addition of glacial acetic acid until the evolution of gas stopped (by visual observation of cessation of foaming). The mixture was then evaporated to dryness on a rotary evaporator "in vacuo". The borate produced from the borohydride was removed by adding 10 ml. methyl alcohol and evaporating to dryness. After two more cycles of methanol addition and evaporation to dryness, the mixture was held in an oven at 100-110°C for 10 minutes to evaporate any residual moisture. Ten ml. of acetic anhydride and approximately 0.5 gram sodium acetate anhydride (Mallinckrodt 7372) were added and the mixture was refluxed in an oil bath at 140° C for 20 minutes. After cooling, the flask was removed from condensor and evaporated to dryness on a rotary evaporator. Approximately 15 ml. of dichloromethane were added to dissolve the alditol-acetate derivatives, and the mixture was then centrifuged at 11,000-12,000 rpm for 30 minutes. The supernatant liquid was decanted into a test tube and evaporated to 1 ml. by gently bubbling a continuous stream of nitrogen gas through the sample. Two microliter portions were used for gas chromatographic analysis.

Since the sugar analysis technique employed was modified by Dr. A. W. Anderson (personal communication) for this study from

the method described by Albersheim, 10 separate sugar analyses were performed upon a single straw hydrolyzate to test the accuracy of the modified technique. Although variations were observed (Table 2), the modified technique was employed because it was more convenient and less time consuming. The modified procedure took 4-5 hours compared to at least one working-day with the method described by Albersheim. To obtain more accurate results, all sugar determinations reported in this thesis were performed in duplicate and the results expressed as the average.

Table 2. Precision data of alditol-acetate technique employed in this study.

Sample	Sugar Conc. (g/liter)				
	Arabinose	Xylose	Mannose	Glucose	Total Sugars
1	1.37	1.31	0.69	0.82	4.19
2	1.26	1.27	0.85	0.91	4.29
3	1.21	1.75	0.65	0.74	4.24
4	1.23	1.22	0.89	0.96	4.30
5	1.26	1.37	0.91	0.98	4.52
6	1.23	1.34	0.79	0.99	4.35
7	1.18	1.34	0.73	0.78	4.03
8	1.22	1.28	0.97	0.90	4.37
9	1.24	1.10	0.90	1.00	4.24
10	1.22	1.20	0.93	1.00	4.35
Average	1.24	1.32	0.83	0.91	4.30
Range	(1.18-1.37)	(1.10-1.75)	(0.65-0.97)	(0.74-1.00)	(4.03-4.52)

Fermentation

Fermentation Vessel

The fermentation vessel used in this study was a two liter Bellco Spinner flask (Bellco Biological Glassware, Inc., Vineland, New Jersey) that was modified for continuous fermentation as is shown in Figure 2. This fermentation vessel measuring 13" x 5" had an operating capacity of 1.5 liters. For our investigations, it was equipped with 3 air ports, 2 of which were equipped with influent and effluent tubes for introducing fresh medium and removing spent medium, respectively. Fresh medium was continuously introduced drop-wise onto the surface of fermentation broth through a 1/8" o.d. stainless steel tube. The spent medium and suspended cells were also continuously removed from the system (at the same rate at which the medium was being replenished) from the bottom of the vessel through a long 1/8" o.d. stainless steel tube. The addition and removal of the medium to and from the system was accomplished by 2 polystaltic pumps (Buchler Instrument, Inc., Fort Lee, New Jersey). The fourth port was reserved for the oxygen probe, which continuously monitered the dissolved oxygen in the fermentor.

The oxygen probe employed was a steam-sterilizable membrane probe described by Johnson et al. (25). The probe was connected to

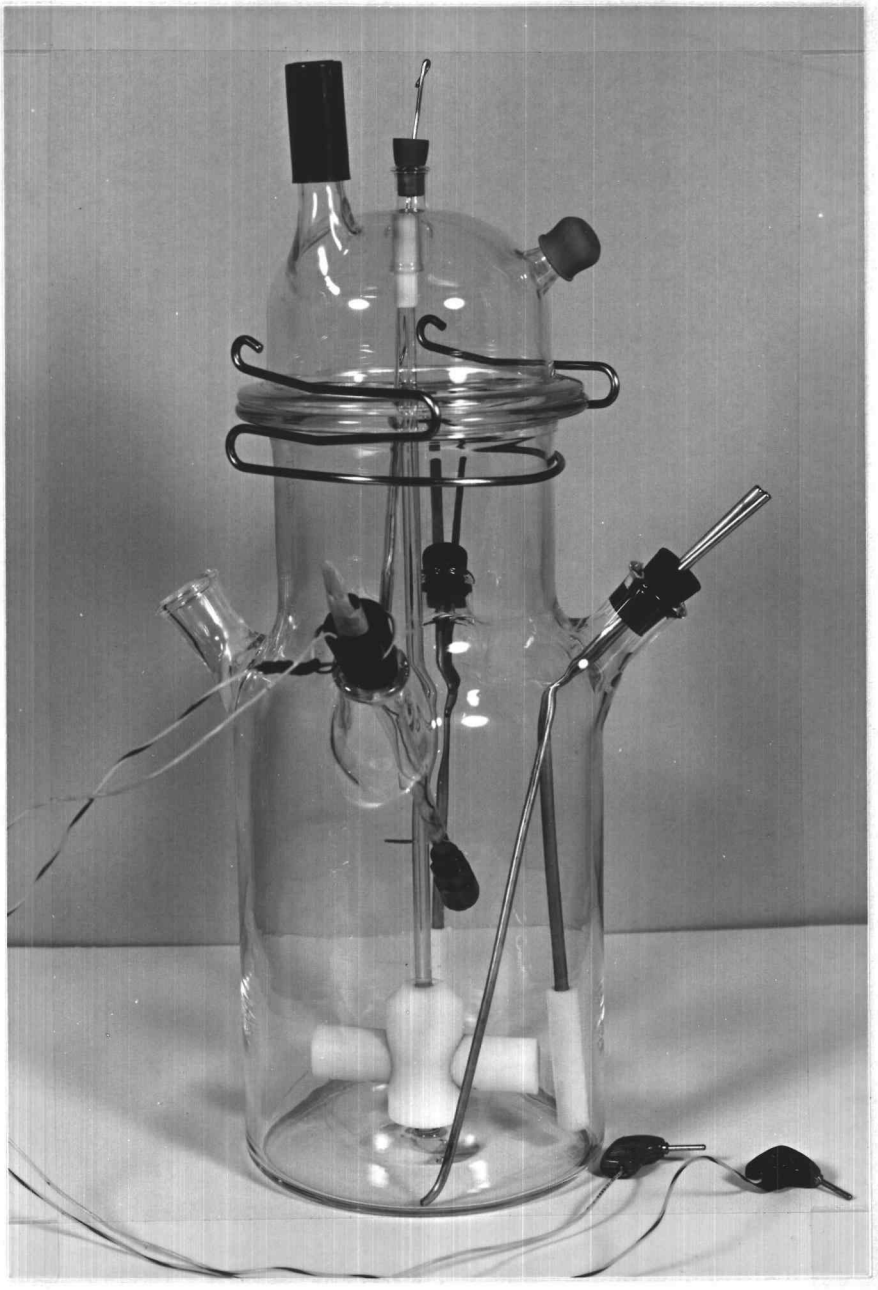


Figure 2. Fermentation vessel.

the probe amplifier and the electrical signals were transcribed on a Rustrak Recorder (Rustrak Instrument Company, Inc., Manchester, New Hampshire).

The calibration of oxygen probe was done with the following gases in pure form and in mixtures: nitrogen gas, 50% nitrogen - 50% air, air, and 50% air - 50% oxygen. The gases were introduced through extra coarse pyrex, fritted cylinder (12 m.m. o.d.) gas dispersion tube at a flow rate of 1 liter/min. into a 3-necked round bottom flask containing freshly boiled water and held in water bath at 32 C. To determine zero chart setting, a stream of nitrogen gas was bubbled through the water in the flask for at least 30 minutes to insure the saturation of nitrogen in the water. The probe amplifier was adjusted until the chart read-out on the Rustrak Recorder was zero. The probe was then transferred into an air saturated flask and the chart read out corresponding to 20.9% oxygen tension in water was recorded. The same procedure was also employed in the determination of percent oxygen tension of 50% air - 50% nitrogen mixture and 50% air - 50% oxygen mixture saturated in water. A standard curve relating the chart read out to corresponding percent oxygen tension in water was constructed (Figure 3).

Agitation of the medium in the fermentor was accomplished by a magnetically driven impeller in the flask. It was operated at the rate of 120-140 rpm. The spinning rate of the impeller was

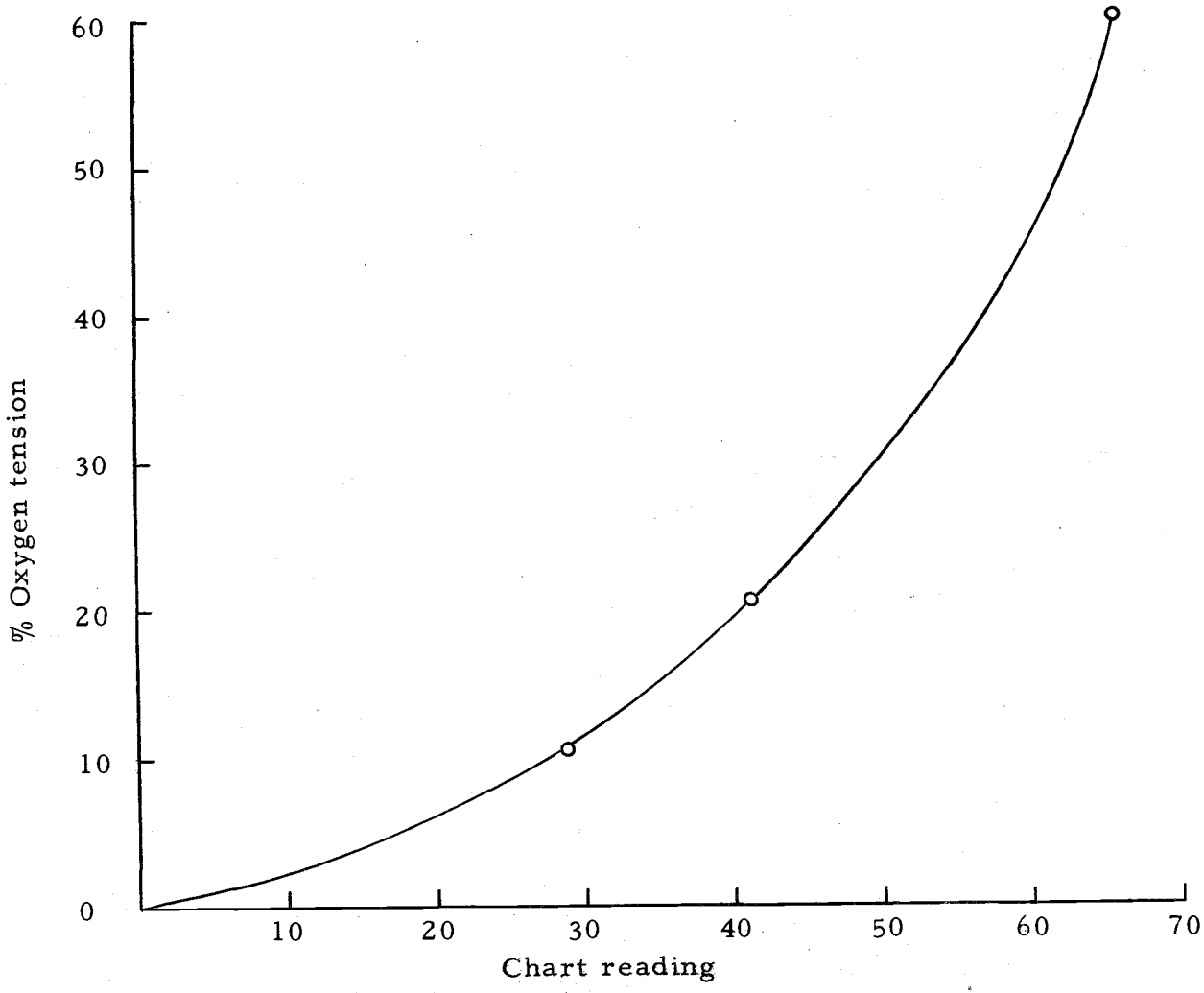


Figure 3. Standard curve of dissolved oxygen percentage.

controlled by a magnetic turntable (5-1/2" diameter) of a bench type fermentor, Fermentation Design "Bio-Kulture" Assemblies (Fermentation Design, Allentown, Pennsylvania) (see Figure 4).

A chemical defoamer, antifoam Y-30 emulsion (Dow Corning Corporation, Midland, Michigan) was used to control excessive foaming using the electronic circuit and pump systems in the bench type fermentor. The fermentation vessel was set in a 32 C water-bath which was placed on the magnetic turntable. The temperature of the water-bath was maintained by continuous circulation of water from a large thermostatically controlled water-bath provided with a small pump (see Figure 4).

Fermentation Vessel Set Up

Fortified straw hydrolyzate was aseptically transferred into the sterile fermentation vessel to obtain an operating volume of 1.5 liters. Candida utilis strain NRRL Y-1084 grown for 18-24 hours in a shaker flask containing straw hydrolyzate, was used as a starter (1% v/v inoculum). Aeration of the fermentor was accomplished by feeding compressed air through sterile glasswool filters at the rate of 6 liters/min. Batch fermentation was continued until the chart read out of the oxygen probe attained a constant value and the optical density (Spectronic 20, Bausch and Lomb, Inc., Rochester, N.Y.) at 600 nm of a 1:10 dilution was about 0.4; this took

Figure 4. Fermentation vessel set up.

A. Refrigerated Influent and effluent media

B. Influent and effluent polystaltic pumps

C. Fermentation flask

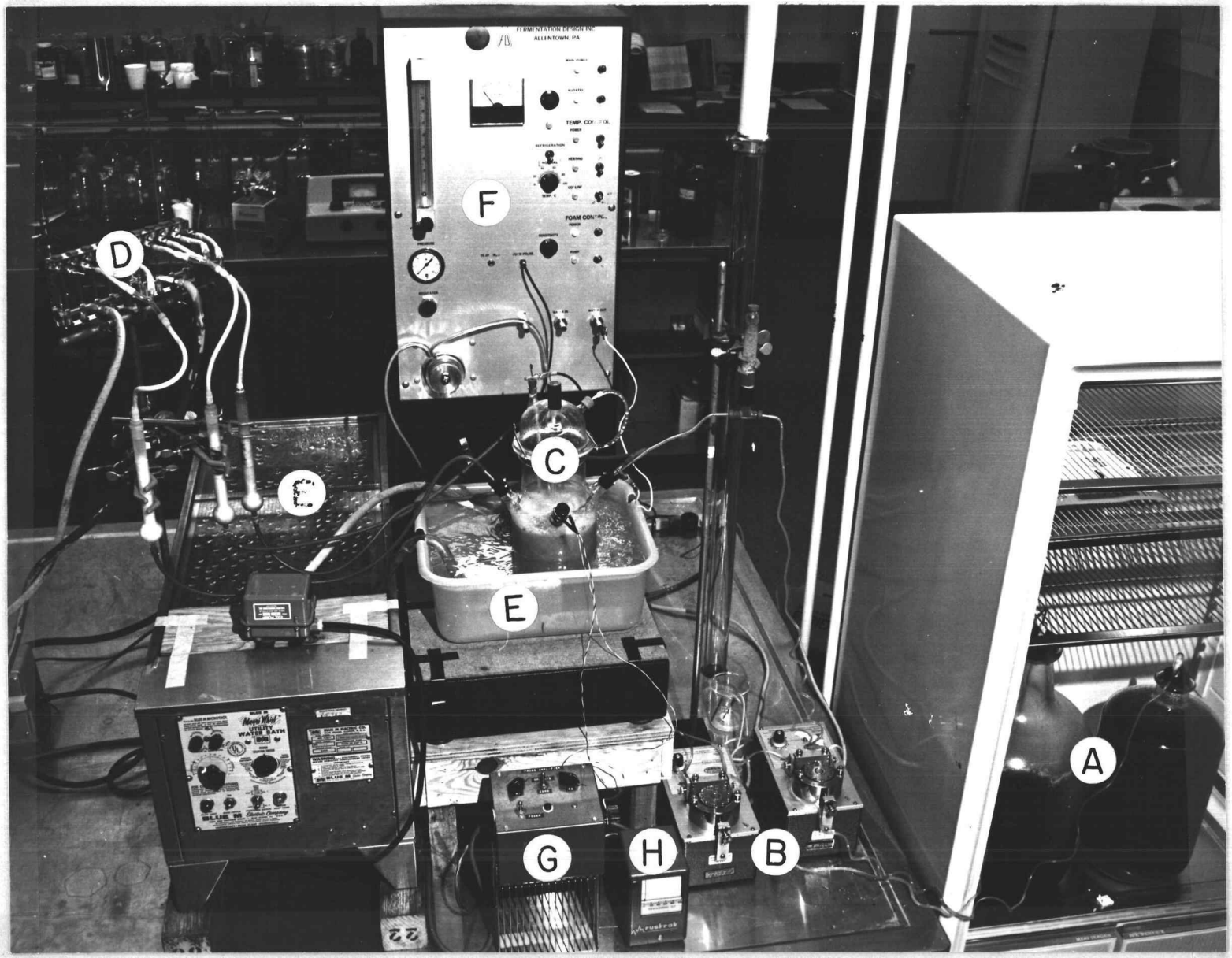
D. Air flow meters

E. 32 C water bath

F. Bench type fermentor base

G. O_2 probe amplifier

H. Rustrak recorder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approximately 12-15 hours. Continuous fermentation was then started. As indicated earlier, the refrigerated medium was continuously fed into and effluent medium removed from the fermentor through the tygon tubing (3/32" i.d., 5/32" o.d.), at the desired rate, by the polystaltic pumps. The pumps were adjusted so that the volume of the medium in the fermentor during operation was kept constant. The spent medium and suspended cells removed from the fermentor were immediately refrigerated to stop or slow down the further assimilation of sugars. In this manner, more accurate analyses of the sugar content in the spent medium were obtained. Figure 4 shows the complete set up of fermentation vessel.

Dry Weight Yield Curve

Cells from one liter portions of effluent broth were collected by centrifugation and then washed twice with 150 ml. of distilled water. The washed cells were lyophilized and weighed. Before cells were harvested, the effluent samples were measured for optical density at 600 nm at different dilutions with a spectronic 20. The dry weight values in three trials were very close. The average values of cell dry weight were plotted against optical density to obtain standard curve (Figure 5).

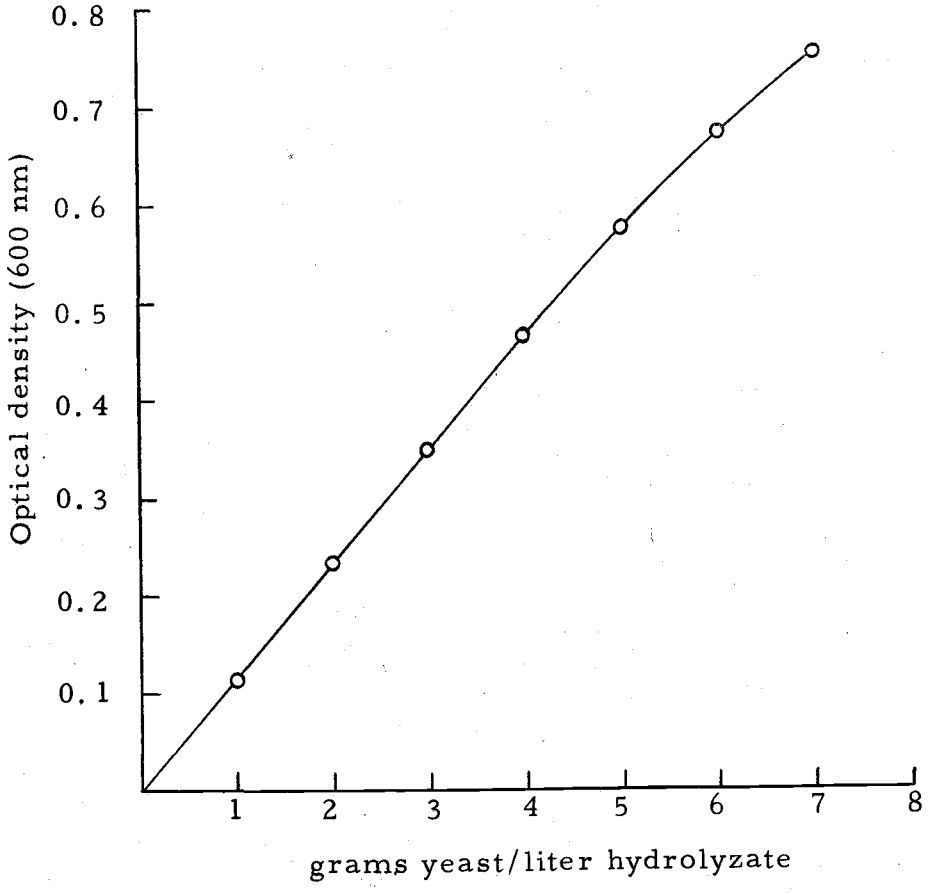


Figure 5. Standard curve of yeast cell dry weight versus optical density.

Aerating Efficiency of Different Types of Air-Dispersion Devices

Three types of air dispersion devices were examined for efficiency in aerating the fermentation broth. The 3 types of aerating devices (Figure 6) were: 1/8" o.d. stainless steel tubes, pyrex gas dispersion tubes (described earlier), and 3/16" o.d. stainless steel tubing fitted with a teflon tip which contained 4 small holes (diameter 13.5/1000") around the bottom. For these studies, air was supplied at a constant rate of 4 liters/min.

Effect of Substrate Concentration

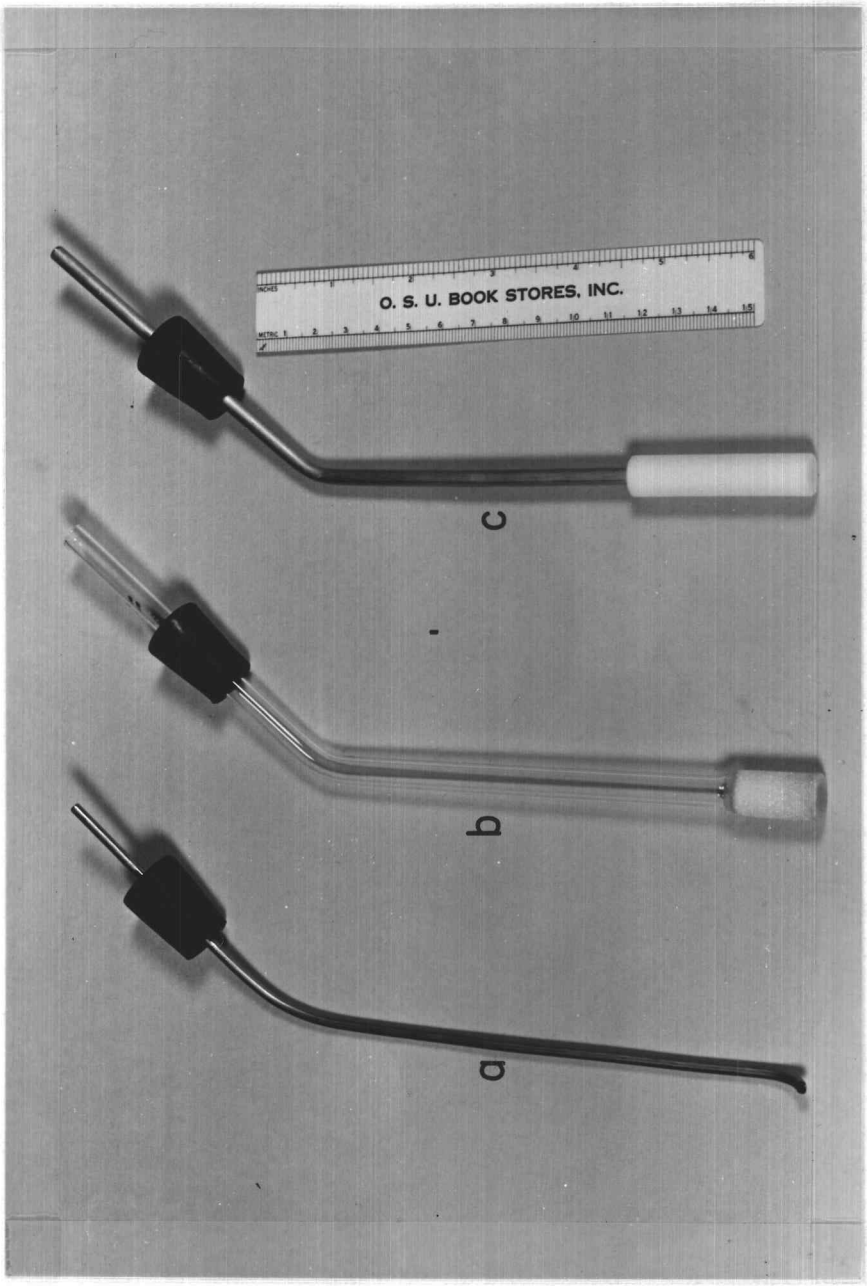
The effect of substrate concentration upon fermentation efficiency was examined by diluting concentrated crude hydrolyzate to contain approximately 6 grams or 12 grams total sugar per liter. During fermentation, the medium addition rate was varied to obtain 12, 9, 6, 5, 4 and 3 hour turnover times (TOT) which correspond to dilution rates of 0.08, 0.11, 0.17, 0.20, 0.25 and 0.35, respectively. Since the influent and effluent pumps were operating at near maximum capacity at a 3 hour TOT, studies operating at TOTs less than 3 hours were not possible. Turnover time is defined as the time required to continuously add 1 fermentor volume (1.5 liters) of medium to the system. Dilution rate is defined as the medium

Figure 6. Air-dispersion devices.

a) 1/8" O. D. stainless steel tube

b) 12 mm. O. D. Pyrex gas-dispersion tube

c) 3/16" O. D. stainless steel tubing fitted with a teflon tip which contained 4 small holes (13. 5/1000" diameter) at the bottom


O. S. U. BOOK STORES, INC.
c
b
a

addition rate in liters/hour divided by the fermentor volume in liters.

At each turnover time studied, effluent samples were analysed for cell density and sugar composition. Effluent samples were taken after the culture had reached the steady state. The culture was judged to be in the steady state when optical density and dissolved oxygen readings were unchanged when observed over a 12-18 hour period. Pump rate, cell density and sugar composition data were used to calculate cell yield rates (grams/hour), sugar addition rates (grams/hour), % sugar utilization and yield constants which is defined as the cell yield (grams) produced per gram total sugar.

RESULTS AND DISCUSSION

The Effect of Heating Time Upon Sugar Release

Arabinose, xylose, mannose and glucose are the four major sugars obtained when straw was subjected to 3% sulfuric acid-heat catalyzed hydrolysis. Work done in this laboratory on acid hydrolysis of ryegrass straw by Frey (17) and also the work of Saeman (42) have shown that the conditions employed in this study would hydrolyze very little, if any, of the acid resistant, alpha-cellulose component of straw; hence, the hemicellulose portion was contributing the four major sugars obtained. Table 3 shows the amount of individual and total sugar released during straw hydrolysis at different heating times. The total sugar yield increased as the hydrolysis time was increased to 45 minutes. The 45 minute hydrolyzate contained approximately 18 grams total sugar/liter. There was little variation in the amount of total hexose sugar detected at different heating times. So, the high total sugar content in the 45 minute hydrolyzate was due to increased amounts of xylose. Degradation of sugars occurred when heating was extended beyond 45 minutes as was evident in the 60 minute sample. As is shown in the table, the decrease in total sugar at 60 minutes was predominately attributable to xylose destruction. This agrees well with work of Saeman (42)

who compared the decomposition rates of sugars released during wood hydrolysis. He demonstrated that xylose was decomposed more rapidly than mannose and that glucose was the most stable sugar.

Table 3. Effect of heating time on sugar concentration of straw hydrolyzate.

Hydrolysis time at 21 C (min.)	Sugar content (grams/liter hydrolyzate)				
	Arabinose	Xylose	Mannose	Glucose	Total
15	0.79	0.32	2.24	2.25	5.60
30	1.57	3.54	2.48	2.31	9.90
45	1.74	12.37	0.78	3.04	17.93
60	1.60	7.80	1.88	2.62	13.90

Since C. utilis preferentially utilizes hexose sugars before pentose sugars (49), the 30-minute hydrolyzate (9.9 grams total sugar/liter) would be more desirable for the production of C. utilis than the 45-minute hydrolyzate (17.9 grams total sugar/liter), because of the higher hexose to pentose ratio.

Aerating Efficiency of Air-Dispersion Tubes

To study aeration efficiency and its effect on cell yield in the fermentation system, stainless steel tubes, fritted glass cylinders and stainless steel tubing fitted with perforated teflon tips were

experimented with as the aerating devices (Figure 6). The effect of dissolved oxygen on yeast yield was monitored for each aeration device. After the culture was allowed to stabilize, the percent oxygen tension in the broth, the optical density at 600 nm and the related cell dry weight were recorded for each type of sparger (Table 4). As expected, a low oxygen tension, 4%, was observed when stainless steel tubes were employed compared to 21% and 16% when fritted glass cylinders and perforated teflon tips were used, respectively. The results also indicated that poor aeration resulted in a low cell yield. Similar results were observed by Johnson (24) when he studied the growth of C. utilis at low oxygen concentrations. The respiration rate of yeast was reported to be directly proportional to oxygen concentration at low oxygen tensions. Thus, when the oxygen supply was limited, carbohydrate was not completely oxidized and was lost as a potential source of energy for the cells.

Oxygen from an air bubble submerged in a fermentation vat must first dissolve in the culture medium and then be transported to the site of respiratory enzymes within the cell. The smaller the air bubbles, the larger the interfacial area and the more efficient the oxygen transfer from the gas to liquid phase. The poor aeration efficiency of the stainless steel tubes was due to their inability to sparge small air bubbles uniformly distributed into the fermentation broth. The fritted glass cylinders and perforated teflon tips

aerated the culture more efficiently because of the uniform sparging of the smaller bubbles throughout the broth.

Table 4. Aerating efficiency of the different air dispersion devices.

Aerating device	O. D. 600 nm	Cell dry weight[a/] (gram/liter)	% oxygen tension[b/]
Stainless steel tubes	0.42	3.6	4
Fritted glass cylinders	0.45	3.9	21
Perforated teflon tubes	0.45	3.9	16

[a/] Cell dry weight were taken from standard yield curve related to optical density (Figure 4).

[b/] % oxygen tension were taken from the oxygen tension curve (Figure 5).

The oxygen tension or efficiency of aeration was the highest with the fritted glass cylinders, but the cell yield was the same as that obtained when the perforated teflon tips were used. The reason might be that the oxygen concentration of the system had reached the critical point for the system. When the oxygen supply to respiring microorganisms is continually increased, a concentration will be reached, and no further increase in respiration will occur beyond the critical concentration. At this high oxygen concentration, the respiration rate which relates to cell yield is independent of oxygen concentration.

Fritted glass cylinders provided the most efficient aeration

(Table 4), but were not used because they tended to clog up with yeast cells after several days of operation, resulting in poor aeration. Since this did not occur with the perforated teflon tips, these spargers were employed in this study.

Effect of Substrate Concentration

To study the effect of substrate concentration on fermentation efficiency, fortified straw hydrolyzate, containing approximately 6 or 12 grams total sugar/liter was fed into the fermentor at different rates. Representative results for straw hydrolyzates containing 6.12 grams total sugar/liter (medium A) and 11.51 grams total sugar/liter (medium B) are shown in Table 5 and 6, respectively.

Continuous fermentation data for medium A (Table 5) shows that the yield constant, which is the ratio of cell yield rate to total sugar addition rate, was 0.63 for the 9 and 6 hour TOT samples. A yeild constant of 0.63 agrees well with reported yield constant for *C. utilis* grown on other complex media (23, 47, 53) and is indicative of maximal conversion of substrate into cells. The yield constant decreased to 0.61, 0.58 and 0.55 as the TOT was reduced to 5, 4 and 3 hours, respectively. The decrease in yield constant indicates that yeast cells were not exposed to the substrate long enough in the fermentation vessel to allow maximum conversion of substrate into cell components. This was supported by

Table 5. Effect of substrate concentration (Medium A)[a] on fermentation efficiency.

Fermentor turnover time (hr)	Pump rates (liter/hour)	Dry weight[c] cell density (gram/liter)	Cell yield rate (gram/hour)	Total sugar addition rate (gram/hour)	Yield constant	Total sugar utilization (%)	Oxygen[d] tension (%)
9 (0.11)[b]	0.17	3.85	0.65	1.03	0.63	82	14.0
6 (0.17)	0.25	3.85	0.97	1.54	0.63	79	11.5
5 (0.20)	0.30	3.77	1.13	1.85	0.61	75	9.5
4 (0.25)	0.38	3.77	1.41	2.46	0.58	65	7.5
3 (0.33)	0.50	3.40	1.70	3.08	0.55	63	5

[a] Hydrolyzate total sugar: 6.12 grams/liter

[b] Corresponding dilution rate

[c] Taken from standard yield curve (Figure 4)

[d] Taken from standard curve of dissolved oxygen percentage (Figure 5)

Table 6. Effect of substrate concentration (Medium B)[a] on fermentation efficiency.

Fermentor turnover time (hr)	Pump rates (liter/hour)	Dry weight[c] cell density (gram/liter)	Cell yield rate (gram/hour)	Total sugar addition rate (gram/hour)	Yield constant	Total sugar utilization (%)	Oxygen[d] tension (%)
12 (0.08)[b]	0.13	5.65	0.71	1.44	0.49	68.5	8.5
9 (0.11)	0.17	5.65	0.95	1.92	0.49	66	6.0
6 (0.17)	0.25	5.45	1.41	2.88	0.49	61	3
4 (0.25)	0.38	5.14	1.93	4.31	0.45	51	2.5
3 (0.33)	0.50	4.57	2.28	5.75	0.40	41	2.0

[a] Hydrolyzate total sugar: 11.51 gram/liter

[b] Corresponding dilution rate

[c] Taken from standard yield curve (Figure 4)

[d] Taken from standard curve of dissolved oxygen percentage (Figure 5)

the sugar utilization data. As the TOT was decreased from 9 to 3 hours, the sugar utilization was lower.

Decreasing the TOT increased the total sugar addition rate thus causing an increase in oxygen demand. Accordingly, the oxygen tension of the fermentation broth decreased as the TOT was reduced. At low oxygen tensions, anaerobic metabolism may have contributed to the observed decrease in yield constant.

Similar results were obtained with the high sugar medium, medium B (Table 6). Yield constants decreased as the TOT was reduced below 6 hours. The decrease in yield constant was accompanied by a substantial decrease in sugar utilization. The highest yield constant obtained from medium B, 0.49, was low compared to the value of 0.63 obtained from medium A. This lower yield constant may have been due to "carbon loss caused by overfeeding" (36). The exact mechanism for this phenomenon is unknown. In addition, oxygen tension values observed for all TOTs were relatively low. Under these conditions anaerobic metabolism may have contributed to the low yield constant. Another factor that may have affected the yield constant was that more antifoam agent was required to reduce the excessive foaming experienced during fermentation of Medium B. This was probably due to the higher cell density and substrate solids concentration. The increased addition of

antifoaming agent has been reported to limit oxygen uptake by microorganisms (39).

Tables 5 and 6 also show that medium B supported higher cell densities than medium A and, consequently, higher cell yield rates were obtained with medium B. Cell densities approaching those obtained with medium B and yield constants approching those obtained with medium A may have been possible with a medium containing a total sugar concentration greater than 6.12 grams/liter but less than 11.51 grams total sugar/liter.

Tables 7 and 8 show sugar analysis of influent and effluents during fermentation of medium A and medium B. Representative gas chromatographic analyses of sugars in the influent and effluents (9 and 4 hour turn-over time) during fermentation of substrates A and B are shown in Figures 7 and 8, respectively.

Table 7. Sugar content in the influent and various effluents of Medium A.

Sample	Sugar (grams/liter)				
	Arabinose	Xylose	Mannose	Glucose	Total
Influent	0.79	1.81	1.67	1.85	6.12
9 Hr TOT[a/]	0.82	0.28	--	--	1.10
6 Hr TOT	0.85	0.46	--	--	1.31
5 Hr TOT	0.84	0.71	--	--	1.51
4 Hr TOT	0.76	1.38	--	--	2.14
3 Hr TOT	0.86	1.43	--	--	2.29

[a/] Turn over time.

Table 8. Sugar content in the influent and various effluents of medium B.

Sample	Sugar (grams/liter)				
	Arabinose	Xylose	Mannose	Glucose	Total
Influent	1.71	5.26	2.39	2.15	11.51
19 Hr TOT[a/]	1.75	1.88	--	--	3.63
9 Hr TOT	1.74	1.96	--	--	3.70
6 Hr TOT	1.77	2.74	--	--	4.51
4 Hr TOT	1.69	3.92	--	--	5.61
3 Hr TOT	1.77	4.83	0.17	--	6.77

[a/] Turn over time.

Sugar analyses of the medium A fermentation (Table 7, Figure 7) show that mannose and glucose were rapidly assimilated and not detected in any of the effluent samples. Since arabinose was not assimilated, the difference in total sugar utilization at the different turn-over times studied was obviously due to xylose assimilation. As the TOT was decreased, xylose utilization decreased. Candida utilis contains NADP-specific polyol dehydrogenase and NAD-linked polyol dehydrogenase which are the enzymes responsible for converting xylose to xylulose, a sugar assimilated via the pentose phosphate pathway (7). According to Horecker et al. (21), these enzymes are specifically induced by xylose and arabinose and are repressed in cells grown on glucose. This is supported by the work of Valavichyus (49) which demonstrated that xylose was utilized slowly and partially

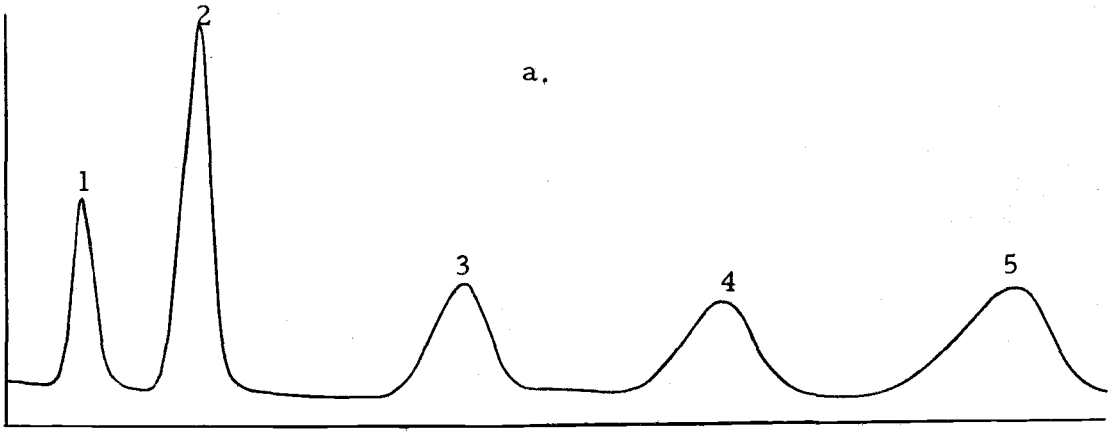
a) Influent medium with sugar concentration of 6.12 g/l.

b) 9 hour turn over time effluence.

c) 4 hour turn over time effluence.

1) Arabinose; 2) Xylose; 3) Mannose; 4) Glucose;
5) Myo-inositol (internal standard)

Figure 7. Gas liquid chromatographic analysis of sugar content in influent and effluent samples of medium A.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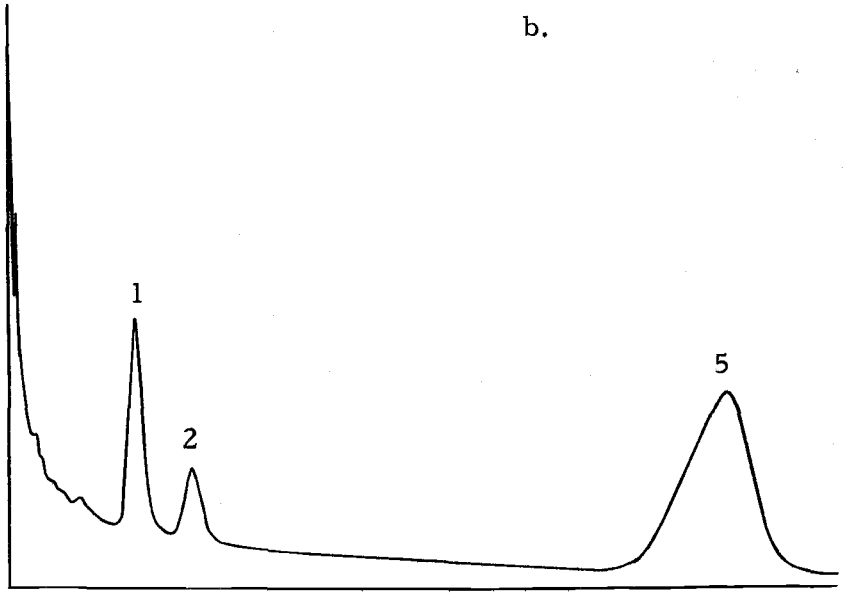
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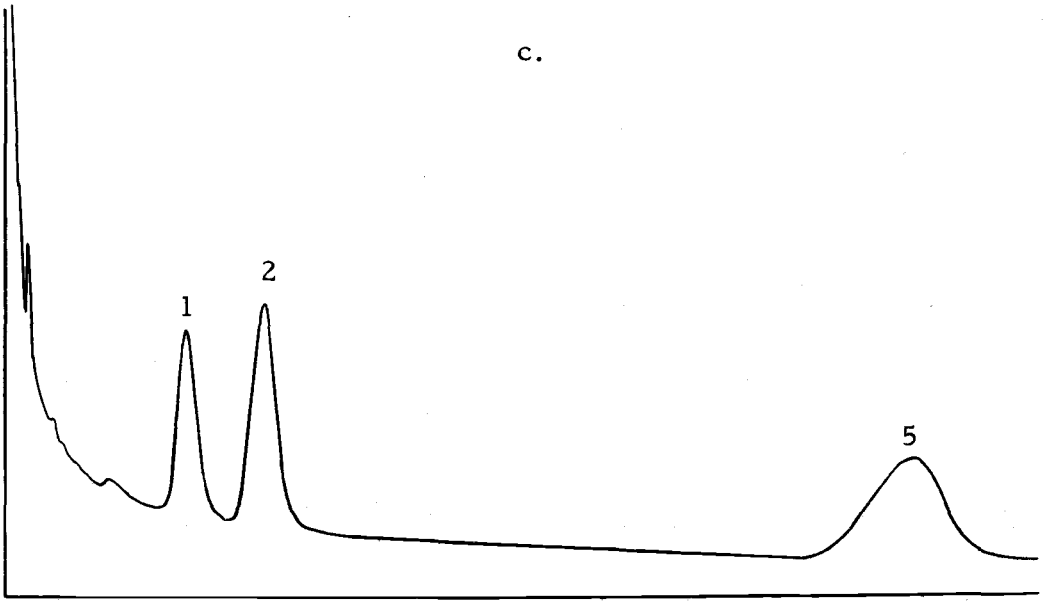
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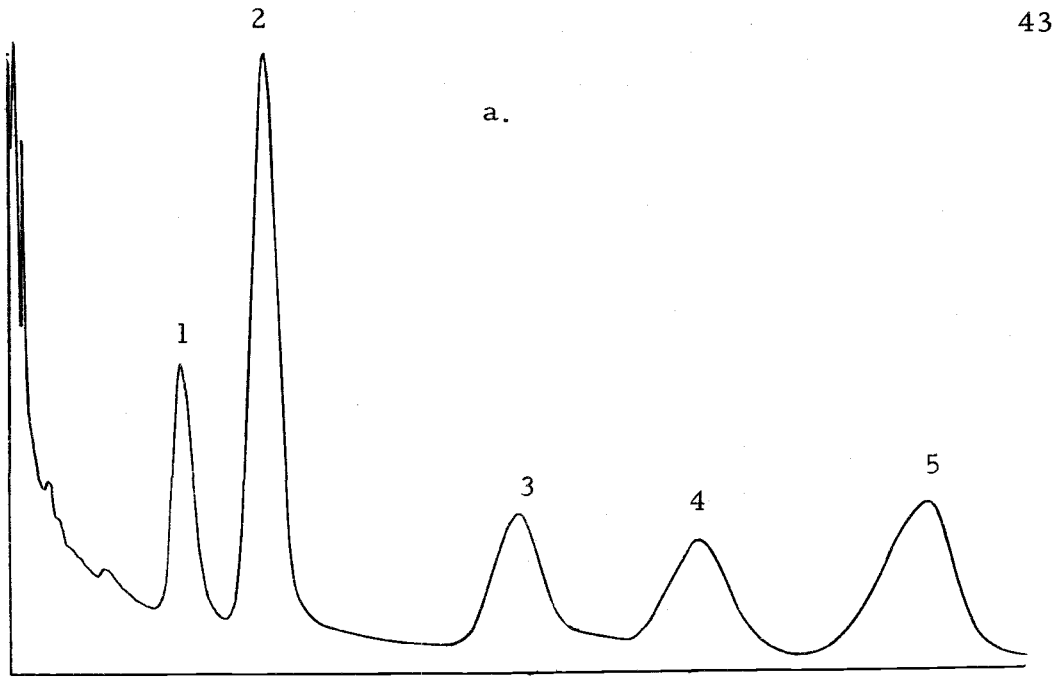
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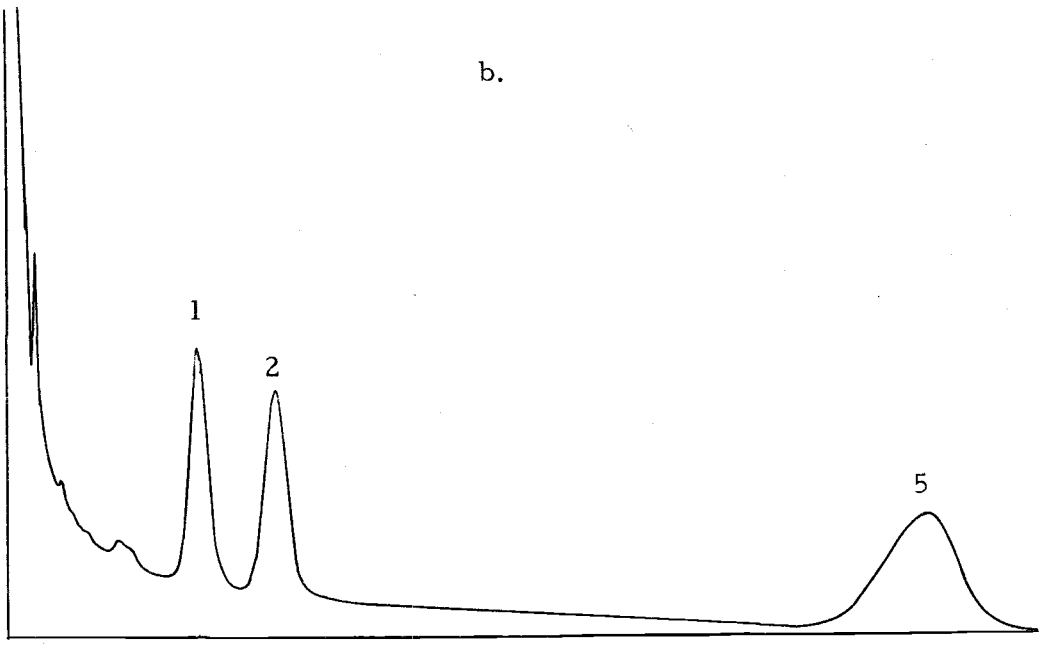
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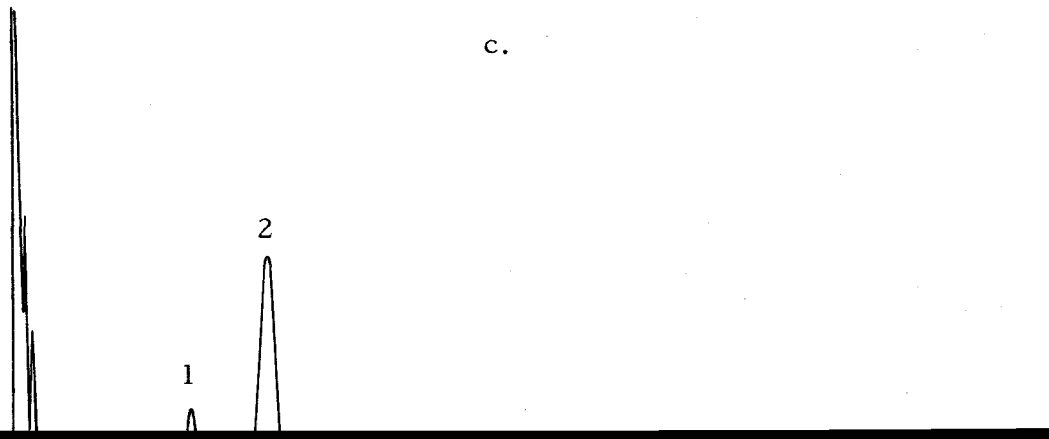
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in media containing hexoses and pentoses, but was utilized 100% and at a higher rate in media containing pentoses only. Since both glucose and the above pentoses are present in straw hydrolyzates, the slow utilization of xylose was probably due to the repression of the two enzymes.

As mentioned earlier, no significant arabinose utilization was detected (Table 8). This agrees with the observation of Lodder (30), but is contrary to the work of others. The polyol dehydrogenases discussed earlier can weakly convert D or L arabinose to ribulose, a readily assimilable sugar. Lack of significant arabinose assimilation can be explained in terms of the low activities of the enzymes responsible for the utilization of arabinose and their repression in the presence of glucose (7, 21). Japanese workers (22) have recently reported conversion of L-arabinose to ribulose by another enzyme in C. utilis called the L-arabinose ketol isomerase. Data pertaining to this enzyme's control and activity have not yet been reported, so a definitive statement about the activity of L-arabinose ketol isomerase in C. utilis cells grown on straw hydrolyzate can not be made.

The lack of evidence for the utilization of arabinose in these fermentations could be because the arabinose was in the D configuration in straw hydrolyzates. This is unlikely since the presence of D-arabinose in other plant hydrolyzates has not been reported. Another possibility might be that the specific strain of C. utilis

(NRRL Y-1084) employed in this study lacked one or more of the arabinose utilizing enzymes. The most plausible explanation is that a longer cell residence time in the fermentor was required for arabinose utilization, especially where hexose and xylose concentrations are not limiting in the system.

The poor sugar utilization observed with medium B, relative to Medium A, was due to high sugar concentration of the medium. The sugar content in medium B was too high to be effectively utilized by the yeast. It is a customary commercial practice to commence yeast propagation in a medium of low sugar content and to add high sugar content medium at such a rate that a low sugar concentration, approximately 0.1%, is maintained in the fermentation broth (36). This condition was approached with the medium A fermentation.

One might expect higher cell yields if the system was operated at TOT longer than 9 or 12 hours. This might not be true in the case of a continuous propagation system where low substrate concentration can become a limiting factor at longer TOT. When the substrate concentration drops below the optimum growth value, the cells pass into a state of cessation (lag). Under these conditions, sugar is used for maintenance and less for growth which results in decreasing yields.

Yeast cells recovered from fermentation effluents and then dried by lyophilization were off-white in color, odorless, and

contained between 49 and 52.5% crude protein based on Kjeldahl nitrogen analysis which was done by the Department of Animal Science, Oregon State University. These protein values exceed the current minimum protein content standards required for food and fodder yeast (37). No difference in crude protein content was detected in cells produced by high TOT (9 hours) and low TOT (3 hours). Finally, extrapolated cell yields of about 150 pounds of dry yeast per ton of straw have been obtained with the system described in this thesis.

In conclusion, the various continuous fermentation parameters studied (i.e., cell production rates, yield constants, and sugar utilization) have shown that annual Ryegrass straw hydrolyzates fortified with simple, inexpensive nutrients can support luxuriant Candida utilis growth. Statements about the economics of yeast production from straw hydrolyzate can not be made from this preliminary study. Such information can be obtained only from pilot plant scale investigations. These future studies will indicate whether or not commercial yeast production from straw is an economically feasible alternative to open field burning.

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