# THE USE OF AMYLOLYTIC MICROORGANISMS FOR THE PRODUCTION OF A HIGH PROTEIN PRODUCT

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ABSTRACT

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Major Professor: R. R. Pereira

A product high in protein content was obtained by fermenting potato starch with a mixed culture of E. fibuliger, A. awamori and C. utilis, or with other mixed cultures of microorganisms. The mash was composed of potato starch, urea, ammonium sulfate, and potassium phosphate adjusted to pH 4.5. Batch fermentation was accomplished at 35°C. for 8 to 10 hours with vigorous aeration and agitation. A 75.9% yield (based on the initial concentration of starch) of product containing 40.6% crude protein was obtained by batch fermentation.

Continuous fermentation by symbiotic growth of E. fibuliger, A. awamori and C. utilis on potato starch was conducted in the same apparatus and under the same controlled conditions. After 67 hours continuous fermentation, the culture yielded a product containing 42.1% crude protein, used 90.4% of the carbohydrate; and gave a 73% yield based on the average concentration of starch in the feed mash. The exchange rate for the continuous fermentation was raised to 17%(V/V) of available fermentor volume; thus the fermentor had approximately four times larger fermenting

productivity daily, based on the same nominal capacity.

The amino acid acid composition of the product showed that the quality of the protein was very close to that of soybean protein. Only 10.7% of the protein could be extracted by water and about 60% remained in the residual fraction after further extractions with salt, ethanol and acetic acid.

An investigation of the carbohydrate changes during the symbiotic fermentation suggests that E. fibuliger excretes $\alpha$-amylase; and that A. awamori and A. niger excrete glucoamylase during the growth in symbiosis with C. utilis or alone.

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# INTRODUCTION

Among microorganisms considered as possible food sources, yeast has attracted perhaps the greatest interest. It represents, in fact, the one sort of microbial protein that has, in the past, been used as a food to an extent that is worth mentioning. As a feed, yeast has had a well established place for many decades. The technology for yeast production is well known, as is its composition and nutritive value.

Candida utilis (formerly called Torula utilis), one of the most noteworthy of the microorganisms considered as a potential food source, has been extensively used industrially for the production of food yeast and animal feed. Since C. utilis is unable to grow on a starchy medium, low cost raw material suitable for growth of C. utilis has so far been restricted mainly to sulfite waste liquors, wood hydrolyzates and molasses. In the past, attempts to utilize starchy plant material, including wastes and surplus agricultural products, as substrates for yeast production have been based principally on methods involving starch hydrolysis with mineral acids followed by a conventional yeast fermentation.

The present study was undertaken to develop a method to convert starchy material into yeast cells by growing C. utilis in symbiosis with an amylase-producing microorganism. Endomycopsis fibuliger and Aspergillus awamori have the capacity to convert starch to glucose, maltose, and other low molecular weight sugars and dextrins, which C. utilis can utilize for its growth and

multiplication. Both batch and continuous fermentations were conducted in the presence of suitable added nutrients, to determine the potential for yeast production. Numerous publications report studies of the enzymic activity of E. fibuliger and A. awamori, but little work has been conducted on the utilization of sugar producing enzymes excreted by these microorganisms to provide a substrate for protein synthesis.

Preliminary attempts were made to identify by paper chromatography the enzymic actions upon the substrate, during the symbiotic fermentation. The quality of the product and the solubility characteristics of its protein were also investigated to evaluate the feasibility of the product for animal feed and/or human food.

# LITERATURE REVIEW

## I. *Candida utilis* and the Yeast Industry

Yeast manufacturing has steadily increased in tonnage, continuing its commanding lead over all other microbial fermentations whose principal end products are living cells.

In the period between 1860 to 1910, yeast was skimmed from grain-malt infusions fermented primarily for their alcohol yield. This dual-purpose, gently-aerated fermentation, the Vienna process, served as the main source of bakers' yeast for as long as the alcohol price was favorable and the supply of grain abundant (1,2).

The discoveries of Pasteur that aerobic conditions stimulated yeast growth and repressed alcohol formation, and that yeast metabolized ammonium nitrogen as well as some organic nitrogen, were slowly explored by the fermentation industry (3,4). Moderate increase in air input, partial replacement of grain wort with beet molasses, souring of mashes, low yeast seedings, and better process control, steadily increased yeast yield at the expense of alcohol yield. From a harvest of 3% dry yield, based on weight of raw materials, in the 1860's yeast yields above 13% were attained by 1915, while alcohol recovery decreased from about 30% to less than 20%(1). Today yeast yields exceed 50% and no alcohol is recovered.

Expansion of the new concepts by Hayduck(5) and Sak(6) launched the modern yeast industry, especially in Germany and the United States. Sak's patents(7) set the pace for industrial yeast developments elsewhere. The lively patent race which followed has been well-documented by Wagner(8).

Throughout the world, yeast has usually first been a by-product of breweries and distilleries. Modern yeast technology began its most rapid growth nearly fifty years ago(9). It was sparked by the wartime shortage of grain and a simultaneous increase in the need for proteins and vitamins. At the present time in the U.S.A., Bakers' yeast tonnage (compressed, active dry, and dried yeast) exceeds the total tonnage of all other types of yeast and yeast products.

General description of C. utilis

A number of factors must be considered in the selection of a yeast for industrial production. The yeast should possess high nutritive value, agreeable flavour, biochemical and cultural stability, ability to assimilate a relatively large number of carbon-and nitrogen-containing materials, the ability to grow rapidly with high yield, easy recoverability, and good appearance.

The yeast commonly used commercially is a strain of Candida utilis, known as 'Torula yeast' and often called Torulopsis utilis. Other yeasts used commercially, or studied extensively in the laboratory, include C. arbores, C. tropicalis, C. pulcherrima, and C. reukaufii; Hansenula anomala and H.

suaveolens; Trichosporon pullulans; and, of course, Saccharomyces cerevisiae.

C. utilis is outstanding in its ability to assimilate a wide variety of carbon and nitrogen sources, including galactose and xylose, and acetic, acetoacetic, fumaric, malic, lactic, pyruvic, and succinic acids. This yeast is thus able to utilize hexoses, pentoses, and other constituents found in the acid hydrolyzates from wood or other cellulosic materials. C. utilis grows rapidly, utilizes pentose as well as hexose sugars, and synthesizes its accessory foods for growth from simple compounds, thus enabling its production from raw materials that are comparatively poor culture media. In contrast, S. cerevisiae, utilizes only hexose sugars and is more fastidious than Torula yeast with regard to its sources of protein and the B complex vitamins.

C. utilis is an asporogenous yeast, the biochemical characteristics of which have been described by Lodder (10), Sperber (11), Dunn (12) and others. There are a number of strains of C. utilis. One, known as C. utilis major, produces fairly large cells; another, C. utilis thermophila, grows at higher temperatures than many other yeasts. These characteristics could be of industrial advantage.

Because of its many good characteristics, C. utilis has been widely used throughout the world as one of the major industrial microorganisms to produce single-cell protein; the substrate has varied, but has included such cheap and readily available

materials as spent sulfite pulp mill waste, wood acid hydrolyzates, molasses, fruit juice and even hydrolyzed starch.

The production of food and feed yeast on commercial and laboratory scales

It is generally believed that food yeast was first produced in Germany during World War II, principally by the Waldhof process (13). This process was rapid and continuous, and employed mechanical aeration and foam-breaking equipment. C. utilis was grown at 35°C on sulfite liquor or wood sugars with a yield of 45 to 48% based on the initial sugar content.

At about the same time, commercial production of food yeast from molasses, sugar-cane juice and raw cane sugar was carried out in a plant located at Frome, Jamaica (14). The organism used in this process was C. utilis. In the United States, the food yeast industry was initiated just after World War II. Kurth (16) and Cheldelin (17) studied the production of fodder yeasts from wood sugar stillage on a laboratory basis. They found that it was most important to use small-size air bubbles for the most efficient yeast propagation. A very important description of the industrial scale production of yeast has been prepared by Inskeep et al (18). C. utilis was grown by a continuous process in a huge fermentor with a pre-treated sulfite liquor as raw material. Fermentation was carried out at 37°C. and pH 4.5. The analytical data showed that C. utilis produced commercially,

from spent sulfite liquor, had the following analysis: moisture, 5.9%; ash, 9.1%; crude protein, 47.4%; crude fat 1.0% and crude fiber, 0.8%.

Similarly, food and feed yeasts have been produced in Taiwan, Japan, USSR, Poland, South Africa and other countries; various materials have been used as carbohydrate source. To overcome the problem of domestic protein deficiency, the Chinese Government established in Taiwan a huge yeast plant, with a capacity to produce 40 tons of dried yeast per day. This plant has been fully operational since 1955; C. utilis is the only organism used and molasses is the sole carbon source. In South Africa, a large yeast fermentor has been installed at Compressed Yeast Ltd. of Industria, Johannesburg (15). This fermentor is used to produce various grades of yeast, for baking, and for production of Bantu beer, pharmaceuticals, and protein additives for human and animal feed. In 1963 (84), the total production of dried food and feed yeast around the world was approximately 260,000 tons, including USSR, 147,000 T(ton); USA, 43,000 T; Poland, 26,000 T; Mexico, 19,000 T; Taiwan, 13,000 T; Cuba, 11,000 T; Czechoslovakia, 10,000 T and France, 9,000 T.

Many agricultural wastes or surplus materials have been tried as carbohydrate sources for yeast production, but most of these investigations have been conducted only on a laboratory scale. Nolte et al (19) described the production of fodder yeast from pressed citrus waste juice: C. utilis was grown in citrus

juice supplemented with trisodium phosphate, ammonium sulfate and sodium carbonate. The fermentation was generally completed in 8 hours with a yield of 44.3% to 48%, based on total sugar in the juice. Reiser (20) reported that C. utilis was grown on a substrate containing the protein waste water of potato starch plants. The yeast, C. utilis NRRL-900, was capable of utilizing as much as 40% of the solids of the protein waste water without the addition of nutrients. Tsilensis and Hydrick (21) studied the production of food yeast; they used the neutralized hydrolyzate of olive residue from an olive-oil plant as the source of carbohydrate. The olive residue was hydrolyzed with 3.5% sulfuric acid at a temperature of 120°C for 3 hours. After addition of yeast extract or malt extract, the neutralized hydrolyzate served as the fermentation mash. Yields of 50 to 65% of dried yeast, based on the sugar utilized, were obtained with C. utilis as the test organism.

Production of fodder yeast on a continuous basis

The continuous culture of yeast was studied by Harris et al (22). The equipment used was a modification of the Waldhof fermentor, which was used in Germany during World War II for the continuous production of food yeast from sulfite liquors. It consisted of an open tank of 34-liter fermentation-medium capacity, a mechanically driven spinner, 6 inches in diameter, which was operated at a speed of 1,100 r.p.m. and served as a combination

aerator and agitator by drawing the fermentation liquor down through a draft tube and forcing it up and around the sides; a proportioning pump to control continuous feeding; and a stand-by pipe, which was slightly higher than the draft tube, for controlling continuous discharge.

The continuous procedure was initiated when the volume of wet yeast in the fermentor was about 7%, or 1.8% dry yeast. At this point, undiluted wood hydrolyzate, of 4.5 to 5% concentration, was admitted. The temperature of fermentation was maintained at 30 to 32°C. and was controlled by cooling coils and an electric heater. No antifoam agent was required because of the type of aerator used. The yeast product obtained from this process, with C. utilis as test organism, contained 50-58% protein content in a yield of 29.6-52.6% and with a 59-93% reduction of the sugar content of the mash.

## II. Nutritive Value of Torula Yeast

The nutritive value of torula yeast has been studied by a large number of investigators, particularly in Germany, Great Britain and the U.S.A. These studies have been primarily concerned with such subjects as the digestibility of the yeast in human nutrition, its vitamin content when grown on different media, its protein content, its amino acid content, and its value either as a food constituent for children and adults or as a feed supplement for hogs, cattle, chickens, rats and other animals.

### Chemical composition of torula yeast

The composition of dried yeast varies to some degree according to the strain of yeast used and the conditions of growth (23). Representative data for torula yeast, as reported by Inskeep *et al* (18), and for Brewer's yeast (24) are shown in Table 1.1. Total crude protein was calculated by multiplying total nitrogen by the factor 6.25, on the assumption that the average protein contains approximately 16% nitrogen. The results of several studies and reviews (25, 26, 27, 28) have indicated, however, that 8 to 13% of the total nitrogen is in purines; 4% in pyrimidines, 0.5% in choline, 0.5% in glucosamine, and smaller portions in other non-protein constituents. Therefore, only about 80% of the total nitrogen of the yeast cell is actually in the form of protein.

The fat content of yeast is worthy of attention. The usual

ether or hexane extraction methods yield values varying between 1 and 2%. In most studies, the nature of this fat has apparently not been determined (18) although there are some studies which have reported its fatty acid composition and unsaponifiable substances (29).

Table 1.1. Representative Chemical Composition of Torula and Brewer's Yeast (as is basis)

| | Torula | Brewer's |
|---|---|---|
| Moisture | 5.9% | 3.9% |
| Protein | 47.4% | 46.0% |
| Crude fat | 1.0% | 2.4% |
| Crude fiber | 0.8% | 2.2% |
| Ash | 9.1% | 5.7% |
| Carbohydrate | 35.8% | 39.9% |
| Phosphorus | 1.9% | |
| Calcium | 0.9% | |

These studies have indicated that the culture medium affects the fat content, increasing its quantity when the medium is rich in sugars.

A further interesting aspect is the relatively high content of ash in brewer's and torula yeasts. Not much is known about the ash components, although Inskeep et al (18) indicated that about 33% was salts of phosphorus and calcium. Wiley et al (30) indicated that the ash content of torula yeast seems to be directly related to the amount of phosphorus nutrient fed to the yeasts.

The other main component, carbohydrate, is present in

the form of glycogen (25), and crude fiber represents the cell wall substances of the yeast. The largest amount of glycogen is of the acid-soluble form, while mannan and glucan are the main components of the cell walls (31).

The nucleic acid of yeast has been studied intensively because yeast is an abundant source of nucleic acid for biochemical studies. The early work of Levene and Jones (90) suggested that yeast nucleic acid was composed of equivalent proportions of four nucleotides that dissociated on treatment with alkali. The nucleic acid content of yeast is approximately 4%, but it varies with the culture procedure and the medium used. Torula yeast has been commercially used as a source of extracted nucleic acid for pharmaceutical purposes.

Vitamin content of yeasts

From the nutritional point of view, yeasts were first used as sources of vitamins, because they represent one of the richest sources, particularly of the vitamin B-complex group. Table 1.2 contains representative data for torula yeast by Inskeep et al (18) and for brewer's yeast (34). As indicated before, the concentration of most vitamins of the B-complex group is relatively high, a fact that makes yeast unique among protein concentrates of vegetable origin.

Table 1.2. Representative Values for the Vitamin Content of Torula and Brewer's Yeast (mcg/g)

| Vitamin | Torula | Brewer's |
|---|---|---|
| Thiamine | 5 | 50-360 |
| Riboflavin | 45 | 36-42 |
| Pyridoxine | 33 | 25-100 |
| Pantothenic acid | 37 | 100 |
| Biotin | 2 | 5-18 |
| Niacin | 417 | 320-1000 |
| Folic acid | 215 | 15-80 |

Yeast also contains a small amount of vitamin E, and of provitamin D which becomes active only after irradiation (24). Most yeasts except Torulopsis rubia, do not contain vitamin A or even α-carotene. Torula-type yeasts are generally capable of producing high levels of pantothenic acid, but this vitamin is subject to rapid destruction under conditions of final processing (30). Similar conclusions for riboflavin have been reached by Wiley et al (30) and Sure and Easterling (33).

## Amino acid content of yeasts

As indicated in a previous section, the nitrogen content of yeasts varies between 7.0 and 9.0% on a dry weight basis. Several investigators have reported that approximately 80% of the total nitrogen is protein nitrogen. Although the composition of the non-protein nitrogen has been determined, its nutritional implications would merit further investigation. The amino acid composition of yeast protein has been the subject of extensive studies by several workers. Representative values are shown in Table 1.3 (34).

Table 1.3. Essential Amino Acid Content of Yeast and Other Proteins (mg/g N)

| Animo acid | Torula | Brewer's | Casein | Cotton seed | Soybean | Egg |
|---|---|---|---|---|---|---|
| Tryptophan | 86 | 96 | 84 | 74 | 86 | 103 |
| Threonine | 315 | 318 | 269 | 221 | 246 | 311 |
| Isoleucine | 449 | 324 | 412 | 236 | 336 | 415 |
| Leucine | 501 | 436 | 632 | 369 | 482 | 550 |
| Lysine | 493 | 446 | 504 | 268 | 395 | 400 |
| Total sulfur amino acids | 153 | 187 | 218 | 188 | 195 | 342 |
| Phenylalanine | 319 | 257 | 339 | 327 | 309 | 361 |
| Valine | 392 | 368 | 465 | 308 | 328 | 464 |
| Arginine | 451 | 304 | 256 | 702 | 452 | 410 |
| Histidine | 169 | 169 | 190 | 166 | 149 | 150 |

The subject of protein composition takes on the greatest importance if yeasts are to be looked upon as a cheap source of protein to supplement poor-quality proteins or to serve as components of protein-rich foods. Csonka (35) reported that amino acids were present in yeast protein in adequate amounts to supply the requirements of the rat, as specified by Rose (36). Skinner and Muller (37) and others reported that the proteins of yeasts and molds were relatively poor in the sulfur-containing amino acids. Block and Bolling (38) presented data which indicated that the percentage of several amino acids in yeast protein varied with the strain. Fink and Just (39) reported considerable variation in amino acid content of the same type of yeast grown under slightly different conditions. Lindan and Work (27) and Spanyer and Thomas (40) studied the sulfur-containing amino acids in bakers' and brewer's yeasts and found both to be low in methionine and cystine. Recently, Peppler (41) reported the amino acid composition of yeast grown on different spent sulfite liquors. The variation found was relatively small for all amino acids with the exception of cystine.

It may be concluded from the studies on amino acid composition of yeasts that most of the variation is due to selection of strain, although growing conditions can also affect the concentration of certain amino acids. Furthermore, the data show that yeast protein is a good source of lysine and has sufficient quantities of other essential amino acids, such as tryptophan and

threonine, the concentrations of which are low in cereal proteins (42). Yeast protein is, however, deficient in sulfur-containing amino acids.

Nature of yeast protein

Very little attention has been paid to the solubility characteristics of yeast protein. Only Thomas (42-45), to date, appears to have studied the solubility of yeast protein. He reported that two proteins were obtained on aqueous extraction of yeast, the yield being greatest if the extraction was carried out at 35°C. in weak alkali medium. One part phospho-protein to three parts true albumin were found. The phosphoprotein, zymocasein, was insoluble in water, soluble in alkali, and contained 16.2% N and 0.9% S. On hydrolysis the zymocasein of yeast was found to resemble milk casein in N partition and content of histidine, arginine and lysine. The nitrogen was reported as follows: $NH_3$-N 6.9%, humic N 4%, diamino or basic N 26.7%, and monoamino N 60.4%. From the basic N fraction, the following amino acids were recovered: histidine 2.6%, arginine 3.6%, lysine 4.1%. The trytophan content was 1.5%. The albumin, cerevisin, resembled that of peas, differing, however, in its lower content of arginine and a marked richness in lysine (8.1% maximum). Other results of the analysis were as follows: $NH_3$-N 5.9%, humic N 1.7%, diamino acid or basic N 23.7%, and monoamino N 67%, histidine 2%, and arginine 4.4%. The tryptophan content was likewise the highest

## III. Amylases of Endomycopsis fibuliger

The best known of the polysaccharidases are the amylases, which act on starch and glycogen. Wheat amylase was one of the first enzymes to be identified. Amylases are found in many plant tissues and in the saliva and pancreas of animals. Among the known amylases, there are two broad groups, designated $\alpha$- and $\beta$-amylases, respectively.

Alpha-amylases have been isolated in highly purified form from human saliva, human pancreas, hog pancreas, rat pancreas, Bacillus subtillis, B. polymyxa, Pseudomomas saccharophila, barley malt, and Aspergillus oryzae, etc.

Alpha-and gamma-(glucoamylase) amylases are widely distributed in various species, from microorganisms to higher plants and animals, but $\beta$-amylase was previously reported in higher plants only. Recently, however, it was found the E. fibuliger contains $\alpha$- and $\beta$-amylases. Purified $\beta$-amylase has been obtained from barley, wheat, sweet potato, yams, etc.

### The discovery of amylases from E. fibuliger

Although different strains of the species E. fibuliger have been known for years, their ability to attack and hydrolyse starch was recognized only recently. Lindner (46), who originally isolated from spoiled bread a filamentous yeast which he named

recorded, 2.2%. Because of its high tryptophan and lysine contents, cerevisin is a valuable nutrient.

Endomycopsis fibuliger, reported that this organism ferments sucrose strongly, glucose less strongly, raffinose and lactose weakly, and maltose and dextrin not at all. Domobrowski (47) reported that the yeast isolated by Lindner did not produce diastase.

In 1942, Wickerham observed that several unidentified strains of filamenteous yeasts isolated from macaroni and from flour used in the preparation of macaroni, produced extracellular amylase. Through extensive studies, Wickerham et al (48) discovered that E. fibuliger is, under certain conditions, a potent diastatic agent. The ratio of $\alpha$- to $\beta$-amylase was found to be high. Two groups of E. fibuliger strains were studied. One consisted of 6 ATCC strains; the other, of 14 relatively recent isolations. The members of the former group produced more amylase but were less fermentative than those of the latter group.

Enzymatic study of amylases from E. fibuliger

Sánchez-Marroquin and Fitch (49) studied the amylase activity of E. fibuliger, with various commercial flours as substrate. The optimum temperature for growth of the organism was found to be 28°C., but the peak fermentation temperature was 37°C. Optimum pH ranged from 4.0 to 5.5. A 15% starch mash prepared from barley and whole wheat flour was found to be the best substrate for fermentation. Aeration of the fermenting culture, at high aeration rates and with occasional manual agitation, accelerated the hydrolysis of starch. Maximum yield of alcohol was obtained

with rice hulls, flour or whole wheat as substrate and with low agitation, or with rice hulls or rye flour substrate and high aeration rates. The same workers also reported that high surface: volume ratio increased the rate of yeast growth and the hydrolysis of starch, but decreased the yield of alcohol.

Following the discovery of amylase production from E. fibuliger, attention was turned to the enzymic characteristics of the amylases (50). Crude amylase was prepared from E. fibuliger and was purified with ammonium sulfate and methanol. The optimum conditions for the activity of the $\alpha$-amylase were pH 5.5 to 6.5 and 65°C., and for the $\beta$-amylase, pH 4.5-6.0 and 40°C. At 50°C., the maximum stability of $\alpha$-amylase was at pH 5.0 to 6.0, and of $\beta$-amylase a pH 4.0 to 5.0. The purified $\beta$-amylase was rather unstable. Calcium ion was not essential for the activity of the $\alpha$-and $\beta$-amylases but increased their stability. Both enzymes could be precipitated by 50 g ammonium sulfate per 100 ml of solution or by 1.2 volumes of methanol. Saturation with $Mg_2SO_4$ did not precipitate either the $\alpha$- or $\beta$-amylase. Thus, a separation of the $\alpha$- and $\beta$-amylases was not obtained and was thought to be impossible on the basis of temperature, pH and precipitation techniques used.

In 1948, Sánchez-Marroquin and Cavarron (51) reported that amylase from E. fibuliger can hydrolyze corn starch to dextrin and a small amount of glucose. The optimum activity of the amylase was said to be at pH 5.0 and 55°C. The activity was proportional

to the substrate concentration within the range of 3 to 10% starch and equalled that of a 10% malt extract.

Fukumoto et al (52) investigated the amylase activity of 12 strains of Endomycopsis and found one of them to secrete a potent amylase when grown in shaken culture. The purification of amylase by salting out with ammonium sulfate and precipitation with rivanol gave a good yield. It was found that the optimum pH of the purified and uncharacterized amylase for saccharification was within 4.5 to 5.5., and that for $\alpha$-amylase was 5.5 to 6.0. Alpha-amylase was stable in the pH range 5.0-7.5, and the saccharifying amylase in the range 5.0-9.0. The enzyme was unstable to heat and EDTA.

A year later, Fukumoto et al (53) examined again the amylase system of 84 strains of fungi imperfecti. They found that Monoscus purpureus, Neurospora, Oospora lactis and E. fibuliger produced relatively large amounts of uncharacterized amylase and $\alpha$-amylase. The amylases obtained were electrophoretically homogeneous and their properties were compared. The amylases of Neurospora and M. purpureus were saccharogenic and those of O. lactis and E. fibuliger were of dextrinogenic type.

In 1961, Hattori and Takeuchi (54) reported that a purified amylase preparation from E. fibuliger was obtained from the culture filtrate by precipitation with ammonium sulfate, dialysis and precipitation with rivanol. This preparation contained both dextrinogenic and saccharogenic amylases. When it was treated with 0.5 M citrate buffer, pH 5.0, at 30°C., and left

overnight, the dextrinogenic amylase was inactivated and a saccharogenic amylase fraction was obtained. Both dextrinogenic amylase and saccharogenic amylase showed maximum activity at pH 4.8 to 5.0. Raw starches of glutinous rice, non-glutinous rice, corn and wheat were digested more easily than those of potato and sweet potato by the purified enzyme. The purified saccharogenic amylase fraction digested raw glutinous rice starch up to about 56%. The same preparation hydrolyzed both soluble starch and amylopectin up to 95%.

In a Japanese patent (55), the Matsutani Chemical Co. claimed that the addition of polyvinyl alcohol is effective for increasing the production of amylase by aerobic fermentation of Endomycopsis species. E. fibuliger was introduced into a medium containing 10% rice bran treated with 0.025, 0.05 or 0.1% of polyvinyl alcohol. After incubation with shaking at 30°C. for 8 days, the saccharification potency of the broth was 800, 824, or 864 respectively, as compared with 704 for the control without polyvinyl alcohol.

Dreimane (56) reported that E. fibuliger, cultured in a modified Reader medium containing 1% starch as sole carbon source, ceased to grow exponentially after 24 hours. The amount of dry biomass formed reached 4.15 g per liter, its protein content was 56%. Further cultivation resulted in a decrease of the biomass and of its protein content. The culture formed amylase, glucoamylase and dextrinase. At 24 hours, the total amylase level

in the medium reached 10.8 units of amylolytic activity, with 70% amylase remaining in the biomass. The formation and excretion of glucoamylase and dextrinase were lower after 24 hours because 80% glucoamylase and 48% dextrinase formed during this period was in the biomass. The culture growth and enzyme formation were enhanced by intensive aeration of the medium and by increasing the temperature up to 40°C.

## IV. Amylases from Selected Species of Fungi

Fungi produce strong saccharifying enzymes which differ significantly from the β-amylases of cereal grains. The molds A. oryzae and A. niger have been used predominantly in the U.S.A. and other countries. Their enzymes catalyze the cleavage of α-1,4 glucosidic bonds but in contrast to β-amylase, split successive (not alternate) bonds from the nonreducing end of starch chains. The products are consequently glucose and dextrins of varying molecular weight. In this thesis the trivial name glucoamylase will be used to describe this type of enzyme. Clucoamylase completely degrades both amylose and amylopectin to glucose, which indicates that the enzyme can hydrolyze the 1,6-glucosidic linkage at the branch point.

In addition, fungi produce strong dextrinizing enzymes, which have been used in the Orient to liquify mash for wine and liquor fermentations.

Glucoamylase has optimum activity in the pH range from 4 to 5 and an optimum temperature in the range of 50 to 60°C, for times up to 24 hours. Glucoamylases isolated from A. niger, A. oryzae or R. delemar definitely and quantitatively hydrolyze maltose; the glucoamylase of A. awamori, however, does not (57). Enzymatic hydrolysis of starch by glucoamylase proceeds entirely by splitting of the bond between the anomeric carbon atom of the glucone and the glycosidic oxygen, while acid hydrolysis leads to a 78% split at the bond between the anomeric carbon atom of the

glucone and the glycosidic oxygen and a 22% split at the bond between the aglucone and the glycosidic oxygen (91).

Enzymatic studies and production of amylases from Aspergillus species

From 1951 to 1956, Okazaki carried out a series of studies (58-67) on these enzymatic reactions and the production of amylases from Aspergillus species. About 300 strains of five different types of molds, A. oryzae, A. usamii, A. awamori, R. tonkinensis and R. pe'ka, were identified for their ability to hydrolyze starch. All strains were found to produce $\alpha$-amylase, $\beta$-amylase (this should be called glucoamylase now) and maltase, but varied in the degree of enzymic activity. The ratio of enzymic activity of $\alpha$-amylase, $\beta$-amylase and of maltase in five types of molds are shown in Table 1.4.

Table 1.4. The ratio of enzymic activity of $\alpha$-amylase, $\beta$-amylase and of maltase in five types of molds

| Type | Strains | $\alpha$-amylase | $\beta$-amylase | Maltase |
|---|---|---|---|---|
| A. oryzae | S4-15 | 100 | 100 | 100 |
| A. usamii | R15-0635 | 32 | 103 | 640 |
| A. awamori | BL5-9 | 110 | 81 | 570 |
| R. tonkinensis | R6-4 | 30 | 98 | 570 |
| R. pe'ka | R2-4 | 5.6 | 47 | 170 |

Okazaki also found that the amylase of A. oryzae type and A. awamori type converted starch to fermentable sugars up to 40 to 50% at a very slow rate, while those of A. usamii, R. tonkinensis

and R. pe'ka types produce fermentable sugars up to 80 to 90% very rapidly and smoothly. The major reason for such a difference, Okazaki explained, would be due to the different ratios of $\alpha$- and $\beta$-amylase in these five types of mold amylase systems. However, Okazaki, found the $\beta$-amylase from mold species were different from barley $\beta$-amylase.

A partially purified preparation of the saccharogenic amylase of A. oryzae was isolated by Okazaki (63) from Takadiastase. The preparation was obtained by saturating Takadiastase solution with ammonium sulfate. After dialysis, the supernatant was allowed to react with 1-2% $HgCl_2$ at 30°C. for one or two days to destroy other enzymes. The saccharogenic activity of this preparation was found to be associated with maltase activity, and the two could not be separated chemically. The optimum pH of this purified enzyme was between 4.2 and 5.2 with an optimum temperature 50 to 55°C. The enzyme lost its activity completely at pH 3.8 to 6.5 within 10 minutes at 70°C. Calcium ions appeared to have an unfavorable influence upon the stability of the enzyme. $HgCl_2$, $CuSO_4$, $Pb(CH_3COO)_2$, $FeSO_4$, $AgNO_3$, $ZnSO_4$, $CaCl_2$, $BaCl_2$, NaCl, NaF, KCl, KCN, phenol, streptomycin, chloromycetin and phenylhydrazine showed neither inhibitive nor activating effects. The enzyme appeared not to be precipitated by trichloroacetic acid, phosphomolybdic acid, phosphotungstic acid, picric acid or tannic acid. About 70 to 80% of the enzyme activity remained in the supernatant when the enzyme solution was saturated with ammonium sulfate. The molecular weight

of the enzyme was 7,500-8,000 by Northrop-Anson's diffusion method. Electrophoresis showed that the preparation contained two components. One of them was thought to be a polysaccharide composed of glucose and xylose; while the other was proposed to be a polypeptide containing tryptophan, arginine, isoleucine, valine, glutamic acid, lysine and one more unidentified amino acid. Furthermore, Okazaki (64-65) purified this preparation by the $Na_2CO_3$-rivanol method to eliminate the polysaccharide and some other impurities, and about a 13-fold increase in enzyme activity was obtained. When compared with the partially purified preparation, the $Na_2CO_3$-rivanol purified enzyme appeared to be precipitated by trichloroacetic acid, basic lead acetate and the saturated ammoniun sulfate. The absorption of the enzyme on active carbon and its decreased stability against pH, temperature and $HgCl_2$ were also observed. The author suggested that some structural change in the enzyme molecule or the elimination of some impurities from it, might be achieved by use of the $Na_2CO_3$-rivanol method.

The mode of reaction of the purified enzyme was also studied. By means of tracer and paper chromatographic techniques, Okazaki showed that the enzyme attacked the starch molecule from the end of the glucosidic chain. Glucose was the major end product but some maltose was also formed. Paper chromatography showed that this enzyme had neither transglucosidic activity upon maltose nor hydrolytic action on isomaltose.

The effect of transglucosylase activity of this enzyme and

yeast on the conversion of starch was also studied. As one would expect, the removal of glucose and maltose from the actively fermenting system changes the equilibrium between the fermentable sugars and starch in the direction of fermentable sugars. Okazaki,s experiment (67) was carried out by using fungal amylase, $\alpha$-amylase and trans-glucosylase on 10% starch solution at pH 5.0 and 30°C. In the first case, only a mixture of $\alpha$-amylase and glucoamylase was used to produce fermentable sugars. In the presence of yeast and in the beginning of the experimental period, the amount of sugars formed was high compared with the amount of sugars actually fermented. Toward the end of the fermentation, all of the sugars capable of being produced were fermented. However, the amount of sugars present in the presence and absence of yeast was almost identical. In the second case, the same amount of $\alpha$-amylase and glucoamylase was used but a fungal transglucosylase was also added to the mixture. The production of fermentable sugars in the absence of yeast was severely limited and actually diminished after about 20 hours. In the presence of yeast, the amount of sugars formed was as high as in the first case, which indicated that addition of the transglucosylase had not diminished the yield of fermentable sugars. This was explained that the reversibility of the transglucosylase reaction shifted the equilibrium toward the production of fermentable sugars while these were removed from the solution by the active alcoholic fermentation.

The amylase of A. oryzae was also found to hydrolyze potato starch, soluble potato starch, amylopectin, amylose, glutinous rice starch, glycogen, $\alpha$-limit dextrin, $\alpha$-$\beta$-limit dextrin, maltose

and panose, provided sufficient time was allowed. The substrates were found to be hydrolyzed up to 100% of the theoretical glucose value. However, the hydrolytic activity of the enzyme on panose was very weak as compared with that of the glucoamylase produced from Rhizopus delemar.

Sadir (68) reported that amylase was obtained from a culture of A. niger NRRL-337 grown in a medium consisting of soybean flour 2%, corn starch 0.2%, malt 0.2%, $CaCO_3$ 0.2%, agar 2.5%, pH 5.5 at 28°C. for 72 hours and then inoculated into a medium containing sorghum hydrolyzate (HCl) 20%, corn starch 1.5%, malt 0.5% and soybean flour 0.5%, pH 5.5 at 28°C. and cultured for 24 hours with agitation. Maximum amylase and maltase activities were obtained after 24 hours.

Watanake and Fukinbara (69) reported that a large amount of saccharogenic amylase was produced by A. awamori in a submerged culture on a medium composed of wheat bran 3.0%, sweet potato starch 1.5%, rice bran 0.5%, $NaNO_3$ 0.2% and HCl 0.025% (initial pH 4.6). For A. awamori, the amount of saccharogenic amylase produced from the submerged culture was about 2.5-fold larger than that produced by the solid culture. It was also found that starch was saccharified only to a saccharification ratio of 95% with a culture filtrate of A. awamori, because maltose-oligosaccharide transglucosylase was present in large amounts in the filtrate. Accordingly, commercial methods for separating transglucosylase from amylase in the culture filtrate were examined. Transglucosylase was absorbed on acid clays,

Amberlite IR-20 or Amberlite IRG-50. Of these methods, use of acid clay was the most effective for commercial elimination of transglucosylase. When starch was saccharified with a culture filtrate treated with acid-clay (3% by volume of culture filtrate) at pH 4.5, the saccharified solution contained more than 98% glucose and less than 1.5% oligosaccharides.

Cadmus et al (70) reported that a high-potency amyloglucosylase was produced from mold of the Aspergillus genus. A. awamori NRRL-3112 and A. niger NRRL-337 were cultured in stainless fermentors with an aeration rate of 1 volume/volume/minute at 600 r.p.m. in a medium of 2 kg of corn meal, 40 g of gibberellin barley malt, and 8 l of water, with the pH maintained at 5.5. Sterilization of the mash and inactivation of the barley malt enzymes was achieved by heating at 60°C. for 20 minutes. Each fermentor was inoculated with 800 ml of inoculum and the medium fermented for 4 days at 35°C. The inoculum was grown on a medium of 5% corn and 0.25% yeast extract. A. niger 337 produced 3.0 to 3.5 amyloglucosidase units/ml and A. awamori 3112, 10-12 units/ml (one amyloglucosidase unit is the amount of enzyme necessary to form 1 g of glucose from 4 g of starch substrate in 1 hr. at 60°C.). A. awamori 3112 produced 3 to 4-fold as much amyloglucosidase and less transglucosylase. Transglucosylase was determined by incubating 0.5 M maltose with A. niger 337 and A. awamori 3112 at 60°C. for 24 hours. Trimethylsilyl derivatives of the carbohydrates were prepared and separated by gas chromatography. In 24 hours, A. niger 337 exhibited an incomplete

maltose hydrolysis and a large amount of isomaltose, but A. awamori 3112 showed nearly complete conversion of maltose and only slight isomaltose production.

Feuiksova and Shilova (71) compared A. awamori with A. oryzae, A. niger and also with R. japonicus, by culturing them on various substrates and employing them in fermentation mashes. It was found that A. awamori showed all the amylolytic and proteolytic activities of the molds used in the fermentation industries, but that its assay with respect to dextrinase was always especially high. The actual number of units obtained depended upon the culture conditions, but A. awamori would always exhibit approximately 3 times as great a dextrinase activity under otherwise similar conditions.

Ohga et al (72) studied some properties of the glucoamylase of A. oryzae cultured on rice. Of 4 amylase preparations from A. oryzae, A-3 and B-2 were electrophorectically homogeneous. The molecular weight, by the sedimentation method, was 69,000; and the isoeletric point was pH 3.3. All 4 preparations showed a pH optimum at 4 to 6, and approximately the same temperature stabilities.

Zherebtsov and Pankratov (74) found that the amylases which occured in malt and in molds such as A. awamori were not markedly different from each other. The apparent differences were due to the presence of melanoidins. The $\alpha$-amylase of both malt and A. awamori are very sensitive towards the pH of the medium. If the pH drops to 4.5 and lower, inactivation occurs; melanoidins

do not inactivate $\alpha$-amylase. The $\beta$-amylase is inactivated below pH 5 because of the pH and the inhibition by melanoidins. The glucoamylase, which occurs in A. awamori in large amounts, is affected neither by acids nor by heat. Melanoidins behave towards it as inert ingredients which simply create a physical dilution effect.

Sep (73) cultured A. awamori 1253 in a medium containing glucose, maltose, sucrose, starch, molasses, $NaNO_3$, ammonium sulfate, peptone and growth promoters, such as malt extract, maize extract or yeast macerate. Such cultures were subdivided into batches of 5000 ml, then fermented at 30°C. for 72 hours; the starting pH was 6, and each batch was stirred at 420 r.p.m. for the purpose of passing air through such submerged cultures. Thereafter each batch was filtered, the filtrates were concentrated and precipitated with 4 times their volume of ethanol. Glucoamylase 3.6 g, was obtained from each batch, with an activity of 54 units/g as determined with a starch substrate.

Smiley (75) patented a procedure of producing amyloglucosidase from mold. This patent describes a dispersion containing ground corn meal 20 parts by weight, water 79 parts, and barley malt 1 part that was acidified by the addition of HCl or $H_2SO_4$ to pH 5.5. The culture medium was heated at 60°C. for 20 minutes to hydrolyze partially the corn starch and lower its viscosity. Then, it was steam-sterilized at 120°C. to inactivate the malt enzymes and to provide a sterile medium. The cooled medium was inoculated with an aqueous suspension of A. awamori NRRL-3112

and was incubated at 35°C. for 4 days with constant aeration and agitation. Removal of the cells provided a liquid containing 12-18 amyloglucosidase units per ml and substantially free of transglucosylase.

Spuller (76) reported the production of amyloglucosidase by molds. A. niger 1276 (NRRL 337), A. niger 1251 and A. awamori 1253 produced 0.10 to 0.70 units of amyloglucosidase per ml of a medium containing 6% soluble starch, Czapek salt nutrients, and 0.5% malt germ extract adjusted to pH 6.0. On raising the starch concentration to 8, 10 or 15%, fermentation liquors showed activities of 1.6, 1.8 or 2.3 units per ml with A. niger 1251 and 1.0, 1.1 or 1.4 with A. awamori. It was reported that in order to reduce cost, potato starch liquified with acid or β-amylase can be used instead of soluble starch, and 1% corn mash instead of malt germ extract. For the prevention of bacterial infections, addition of 300 μg of chloramphenicol/ml or of 20 μg of oxytetracycline/ml was found to be suitable.

Kursheva and Fedorov (77) studied the saccharification of starch and acceleration of fermentation by Aspergilli. Strains of A. oryzae and A. awamori were developed to complement each other so that a mixture of their crystallized enzymes would hydrolyze starches. Mixtures of natural barley malt plus glucoamylase from A. awamori may also be used. In order to exploit the enzymes at their optimum temperatures, enzymes mixtures were incubated at two different temperatures. Thus, the enzyme mixture was

incubated at a substrate concentration of 4%, a pH of 5.3, a first temperature of 58-59°C. and second temperature of 32°C.

## V. Yeast Fermentation by a Symbiotic Process

### Alcohol fermentation by a symbiotic process

Wickerham et al (48) were the first to report diastatic activity by different strains of the yeast, E. fibuliger. These authors studied the possibilities of the industrial production of ethanol, 2,3-butylene glycol, and acetylmethyl carbinol by using mixed fermentations consisting of the amylase-synthesizing organism, E. fibuliger, together with Saccharomyces cerevisae or Aerobacter aerogenes. The study demonstrated that amylase production in aerated wheat mashes was quite low, while high yields of $\alpha$-amylase were obtained on a porous substrate such as bread. When E. fibuliger was used alone in the saccharification and alcoholic fermentation of wheat mash, bread cultures of various strains of Endomycopsis produced from 13.2 to 54.0% of the theoretical yield of alcohol; when such cultures were used in association with S. cerevisiae, 63.7 to 78.3% of the theoretical yield of alcohol was obtained. The use of Endomycopsis cultures for hydrolysis of starch in the butylene glycol fermentation gave total yields of butylene glycol, ethyl alcohol, and acetylmethyl carbinol equivalent to the yields obtained with malt-saccharified mash. The amount of alcohol produced in the mashes saccharified by Endomycopsis, however, was greater than the amount produced in malt-saccharified mash.

Sánchez-Marroquin and coworkers (50, 51) studied the use

of a mixed fermentation using E. fibuliger and Streptococcus carbajali for the production of ethanol from starchy substrates. It was found that the mixture gave the best starch hydrolysis and fermentation in 10% premalted wheat mash at pH 4.5-5.0 with 3-minute agitation every 24 hours. Temperatures from 28 to 37°C. were beneficial for the growth and activity of the cultures. A temperature of 45°C. inhibited the growth. The fermentation yield was 84.6% ethanol, 3 mg % acetic acid, 0.8 mg % furfural, and 200 mg % amyl alcohol for the mixed culture and 40.9% ethanol for E. fibuliger alone. Rice mash gave similar results, while barley, rye and banana mashes were less satisfactory. An inoculum of 3 ml of S. carbajali to 5 ml E. fibuliger were best for 250 ml fermentable medium.

Yeast culture in a symbiotic process

Numerous workers have reported studies of the enzymatic activity of E. fibuliger and A. awamori. However, little work has been reported on the utilization of sugar-splitting enzymes from these microorganisms for protein biosynthesis. Kuehner (78) was the first to use amylolytic yeasts for the production of a nutritional product. In his Ph.D. thesis, he described how a mixed culture consisting of an amylase-producing yeast, either E. fibuliger or E. chodati, and a non-amylase-producing yeast such as C. utilis, were cultured on cooked, but not converted, starch mash such as wheat, corn, rice or potatoes.

Similarly, Kuehner and Wickerham (79) described a process in which a mixed culture consisting of an amylase-producing yeast (either E. fibuliger or E. chodati) and a non-amylase-producing yeast (such as C. utilis) were grown on a cooked, but not converted, starch mash such as whole wheat, corn or potatoes. The entire mass of centrifugable solids, was harvested as the product. This product contained both yeast and the residual material from the substrate used. In general, yields of approximately 60 pounds of solids were obtained from 100 pounds of raw material (dry basis). The protein in these solids varied from about 20 to 35% when wheat was used. When potatoes were used, the percentage of protein in the solids ranged between 21.5 and 36.5. The optimum range of concentration of wheat mash was 5 to 10%. Urea, incorporated in the mash as a nitrogen source, had the advantage of facilitating maintenance of a constant pH and was used in place of ammonium sulfate for this reason. Additives such as magnesium sulfate, calcium carbonate, and potassium phosphate were not necessary under the conditions used. The process operated over a broad range of pH values, the optimum being at pH 5 to 6. The best agitation and aeration rates would appear to be 650 r.p.m. and 1 volume of air per volume of mash per minute under the experimental conditions. The re-use of diastatically active supernatant liquid obtained from a previous fermentation with a starch-hydrolyzing yeast was also studied. Use of this liquid was effective in reducing the initial viscosity of a starch mash by partially saccharifying

the starch. Yields of yeast protein rose slightly in the first recycling and dropped steadily from the second to the sixth recycling.

In 1959, Wickerham (86) was granted a U.S. patent to cover the process described above. This patent reports that yeast preparations high in protein and vitamin content are obtained by culturing of E. fibuliger or E. chodati singly or in combination with other yeasts on amylaceous substances and recovering the insoluble matter. For example, these yeasts were propagated on 5% ground wheat mash, 0.25% ammonium sulfate, 0.25% urea, 0.1% $KH_2PO_4$, 0.05% $MgSO_4$ and 0.001% $CaCO_3$. The mash was adjusted to pH 5.0 and incubated at 33°C. for 8 hours with vigorous aeration and agitation. E. fibuliger and E. chodati are unique as yeasts, in that they contain extracellular amylolytic enzyme systems and hence can be propagated directly on inexpensive amylaceous materials, such as potatoes, cereal grains, and waste from grain-wet-milling plants. Suitable N-sources for the fermentations are corn steep-liquor, distillers solubles, protein hydrolyzates, urea and ammonium and nitrate salts. To provide higher concentrations of protein, other yeasts, such as Torulopsis utilis or Hanenula subpelliculosa, were cultured simultaneously or subsequently to the amylolytic yeasts.

About ten years later, Jarl and Tveit (30) investigated this process again and turned their direction to the removal of starch from industrial waste liquids by symbiotic cultivation

of yeasts. They emphasized that starch is most common in waste liquids from food industries and will often create a water pollution problem, if untreated. Standard biological methods may be used for waste treatment, but it will require an extensive purification plant if the operation is of appreciable size. It was estimated that around 15-20% of the total starch of in-going potatoes in potato processing plants may be lost during processing. Consequently, they conducted an investigation with the aim of converting this waste starch into yeast cell mass by a symbiotic process, where the yeast E. fibuliger through biological hydrolysis will convert the starch to dextrins, glucose, and other low molecular weight sugars which C. utilis will be able to utilize for its growth and multiplication.

Simultaneously, a patent was granted by the U.S. Government to Tveit (81) on the process. This patent claimed that potato starch is fermented symbiotically with starch-hydrolyzing and sugar-fermenting fungi to produce yeast. Thus, 4 kg of sterile medium containing 7.2 g urea was prepared by slurrying 250 g of grated potatoes in water. After adjusting the pH to 5.2, the medium was inoculated with a mixture of E. fibuliger and C. utilis, and fermented for 48 hours at 30°C. while aerating with 40 liters of air/l/hr. After complete fermentation, yeast containing 31% raw protein was obtained at 80% yield (based on the initial quantity of starch). The use of ammonium sulfate instead of urea did not decrease the yield provided the pH was

kept at 5.0 by addition of alkali.

Zelinka and Polivka (82) reported that E. fibuliger was used to convert starch for the production of feed concentrate. A medium containing starch 3 to 6%, corn steep liquid 1 to 3% (dry weight) and $(NH_4)_2HPO_4$ 0.1-0.4% (pH 4.4-4.7) was inoculated with E. fibuliger and fermented for 18 to 30 hours to give a product rich in proteins, vitamins and growth factors in 50 to 70% yield, based on the amount of starch used.

Nordheim et al (83) reported on the production of fodder yeast and food yeast from potatoes by use of enzymic starch saccharification procedures. The saccharification of the starch from potatoes by glucoamylase-containing preparations (alone, in combination with α-amylase, or after pretreatment with α-amylase) provided favorable conditions for optimum microbial biosynthesis by C. utilis. The advantage of the enzymic procedure lies in the formation of a great amount of sugars suitable for yeast production, when compared to the previously developed methods of hydrolysing starch with mineral acids, and also in the fact that other compounds in the potatoes are not known to be broken down. Yeast propagated in enzyme-saccharified potato substrates is preferable to that obtained from acid-hydrolyzed substrates because of the yield and content of amino acid, vitamins, phosphorus and protein.

Jarl (84) described the 'Symba' yeast process in which the production of a microbial food from low cost starch materials

and the purification of industrial waste starch effluents feasible. The major principle of the process was the same as that previously described, except that a continuous process was mentioned. The final product of the mixed culture, E. fibuliger and C. utilis, contained about 7% moisture. The crude protein content varied between 40 and 50% (dry basis) depending on the raw material. Fiber content was about 5%, ash was 5% and lipid was 4 to 5%. Studies of a batch Symba yeast process have been carried out by the Swedish Sugar Corporation on a plant scale. A pilot plant with daily capacity of 100 kg of yeast product is being prepared for test-run. A three-year program is in progress, aimed at translation of the continuous Symba principle to a full scale commercial process.

# EXPERIMENTAL

The experimental work of this investigation will be presented in four sections. Each section is written in the form of a scientific paper that can be submitted for publication with only minor editorial changes. This format was adopted in an attempt to decrease the time lapse between the preparation of the thesis and the preparation of the scientific papers resulting from the investigation.

The first section deals with production of a product of high protein content from potato starch by a symbiotic process. The second section deals with production of a product of high protein content from potato starch by a continuous and symbiotic process. The third section deals with the change in carbohydrates during the growth of yeast in a symbiotic culture, and the last section deals with a qualitative study of the protein from a symbiotic culture.

## Section I. Fermentation of Potato Starch by a Symbiotic Process


### Abstract

A product high in protein content was obtained by fermenting potato starch with a mixed culture consisting of either E. fibuliger or A. awamori and C. utilis, or other combination of these microorganisms. The mash was composed of potato starch, urea, ammonium sulfate, and potassium phosphate and was adjusted to pH of 4.5. Fermentation was carried out at 33-35°C. for 8-10 hours with vigorous aeration and agitation. Product yield was 75.9% based on the initial concentration of starch. The product contained 40.6% crude protein. The symbiotic fermentation of E. fibuliger, A. awamori and C. utilis was found to be the best as far as the control of fermentation or yield was concerned.


### Introduction

This investigation was aimed at the conversion of potato starch into yeast cells by growing C. utilis in symbiosis with an amylase-producing microorganism, E. fibuliger and a gluco-amylase-producing fungus species, A. awamori or A. niger. E. fibuliger and A. awamori or A. niger could theoretically convert starch to glucose, maltose, other low molecular weight sugars and dextrins. C. utilis could conceivably then use these sugars for its growth and multiplication. Since the growth rates of E. fibuliger and A. awamori or A. niger are moderate, the process would yield a product substantially of Torula yeast.

Candida utilis (formerly called Torula utilis), one of the microorganisms often considered as a possible concentrated source of food protein, has been used as an industrial strain in the food yeast industries around the world. As a feed, it has had a well established place for many decades. Unfortunately, C. utilis cannot be grown on a starch medium, due to its lack of diastase activity. Low cost raw materials suitable for production of C. utilis have so far been restricted mainly to sulfite waste liquors, wood hydrolyzates and molasses. In the past, attempts to utilize starchy plant materials (wastes, surplus agriculture products) as substrate for yeast propagation have mostly been based on the principle of starch hydrolysis with mineral acid followed by conventional yeast fermentation.

Numerous publications have already reported studies of the enzymic activity of E. fibuliger and A. awamori or A. niger. Little work has, however, been conducted on the utilization of starch-splitting enzymes of these microorganisms for protein biosynthesis. In this field, Kuehner and Wickerham (79) studied the use of E. fibuliger in mixed culture for the production of feed yeast and patented their process in 1956. Jarl and Tveit (80) studied the same process in Sweden, and Tveit obtained a U.S. Patent in 1964 (81). Both studies were aimed at the conversion of starch into C. utilis cells by a symbiotic process, and E. fibuliger was the only amylase-producing organism used.

The disposal of potato waste is a serious problem in the potato processing industry. Currently, several methods have

been proposed to meet this problem. Standard biological methods may be used to treat potato waste, but will require a costly purification plant, which will yield by-products of little, if any, value. The successful use of the symbiotic culture principle would utilize the major portion of the available starch in potato waste, and reduce the requirement for standard waste treatment facilities for the residual unfermentable materials. Moreover, the mixed product of E. fibuliger, A. awamori, C. utilis and carbohydrate, a product high in crude protein, could be used as a feed supplement. It is hoped that the value of this product may be sufficient to reduce considerably the cost of purification of the waste water by conventional biological methods.

Materials and methods

Whole potatoes were used throughout this study. Unpeeled tubers were washed in cold tap water and ground to a fine state in a Waring Blendor. The mash was adjusted to approximately 4% starch content with tap water. Nutrients for the organisms, viz. 0.05% $K_2HPO_4$, 0.25% urea, 0.25% ammonium sulfate, were added to the potato mash before its pH was adjusted to 4.5 with dilute HCl or $H_2SO_4$. The mash was sterilized by autoclaving at 248°F. for 15 minutes before use.

For a comparative study, the ground mash of unpeeled potatoes was mixed with 3.5% of conc. HCl (35-38%) on a W/W basis and autoclaved for 2 hours at 248°F. The yield of sugar was found to be 15 pounds glucose for each 100 pounds of fresh potatoes

after filtration. The sugar solution was also adjusted to approximately 4% glucose concentration with tap water before use. After adding urea, ammonium sulfate and $K_2HPO_4$ as described previously, the diluted sugar solution was employed as the fermentation mash for C. utilis only.

During the course of this work C. utilis NRRL-900 was cultured on Difco wort agar at 35°C. for 2 days and then kept in a refrigerator (4°C.). The amylase-synthesizing organisms were maintained on slants of a soluble starch-malt extract-yeast agar at 35°C. for 3 days and also kept in a refrigerator (4°C.). The composition of this medium was as follows: 3 g yeast extract, 3 g malt extract, 5 g peptone, 20 g agar, 10 g soluble starch, and distilled water to make 1000 ml; it was sterilized before use. All cultures were transferred at least once per six weeks to maintain the maximum activity of the microorganisms.

Inoculum of the starch-hydrolyzing organisms (E. fibuliger NRRL-Y-25 and A. awamori NRRL-3112 or A. niger NRRL-3122) was prepared by inoculating a 50 ml portion of sterile 4% mash (4% starch, 0.05% $K_2HPO_4$, 0.25% urea, 0.25% ammonium sulfate and 0.01% yeast extract) with 0.5 ml of a suspension washed directly from the pure agar slant culture. The cultures were incubated on a rotary water bath shaker for 2 days at 35°C. The inoculum of C. utilis NRRL-900 was prepared by the same procedure as described previously, except that glucose was used in the medium instead starch. After incubation, the cultures of starch-hydrolyzing

organisms, E. fibuliger and A. awamori or A. niger were added to the fermentor at a 1:1 ratio, at a rate of 10% based on total mash volume. The initial concentration of C. utilis cells added to the mash was at a rate such that approximately 1 x $10^8$ cells per ml of mash would be realized. The level of mixed yeast cells in the mash was determined by direct microscopic count using a Levy Counting Chamber.

All fermentation experiments were carried out in a modified Waldhof fermentor designed and built in our laboratory. The capacity of the fermentor is 17.2 liters with an available fermenting volume of 8 liters. The internal structure of the fermentor is presented in Fig. 2.1. This fermentor consists of an air sparger, air inlet and outlet tubes, rotary plate and draft tube, all constructed of stainless steel. Aeration was achieved by rotating the rotary plate at 600 r.p.m. and introducing filtered air through the sparger at a rate of 1 liter of air per 1 liter of mash per minute.

The pH of fermented mash was controlled automatically by a Chemap pH auto-controller at 4.5. Five to eight liters of the sterile medium described previously was inoculated simultaneously with different combinations of cultures, i.e. E. fibuliger and C. utilis; A. awamori and C. utilis; A. niger and C. utilis; E. fibuliger, A. awamori and C. utilis; and E. fibuliger, A. niger and C. utilis and fermented for approximately 10 hours at 33 to 35°C. The growth of yeast cells was usually completed within 8 to 10 hours.

The yeast was harvested by centrifuging the final fermented mash and washing the cells with distilled water; this was repeated for three centrifugations (3000 r.p.m., 10 minutes) in a Model K, Size 2, International centrifuge. The washed product was dried in a hot air oven (105°C.) for 16 hours. The total carbohydrate content of the mash was determined by direct acid hydrolysis. Sugar determinations were made on the wort and fermented mash by the Shaffer-Somogyi Micro Method (85). The protein content of the dried yeasts was assayed by the official Kjeldahl determination, and corrected for moisture.

Results and Discussion

Since this investigation was concerned only with yield of product and reduction of starch content from potato wort by symbiotic growth of microorganisms, attention was paid mainly to the growth of C. utilis and to the changes in carbohydrate content in the mash during fermentation. The results of yeast growth and changes in carbohydrate are presented in Figures 2.2 to 2.7.

Results of the symbiotic fermentation with different combinations of amylase-producing organisms are presented in Table 2.7. The data indicated that the yield (yeast mixed with amylase producing-microorganisms and residual potato solids), based on the starch utilization, was 72.9-79.5%. The crude protein of the dried mixed product varied from 32.8 to 40.6% and differed from batch to batch and from organism to organism. Yield of yeast cells was found

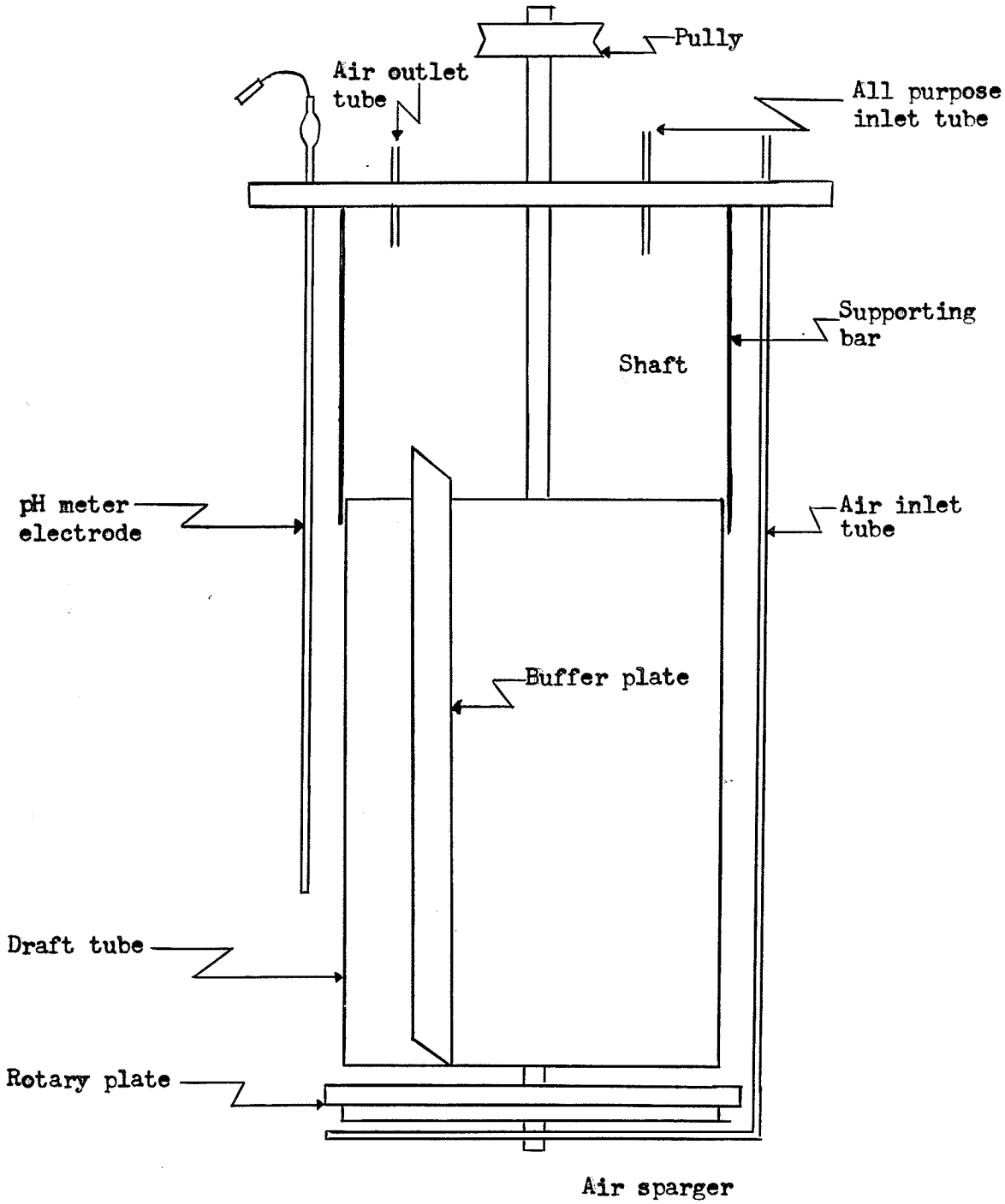


Figure 2.1. Modified Waldhof Fermentor

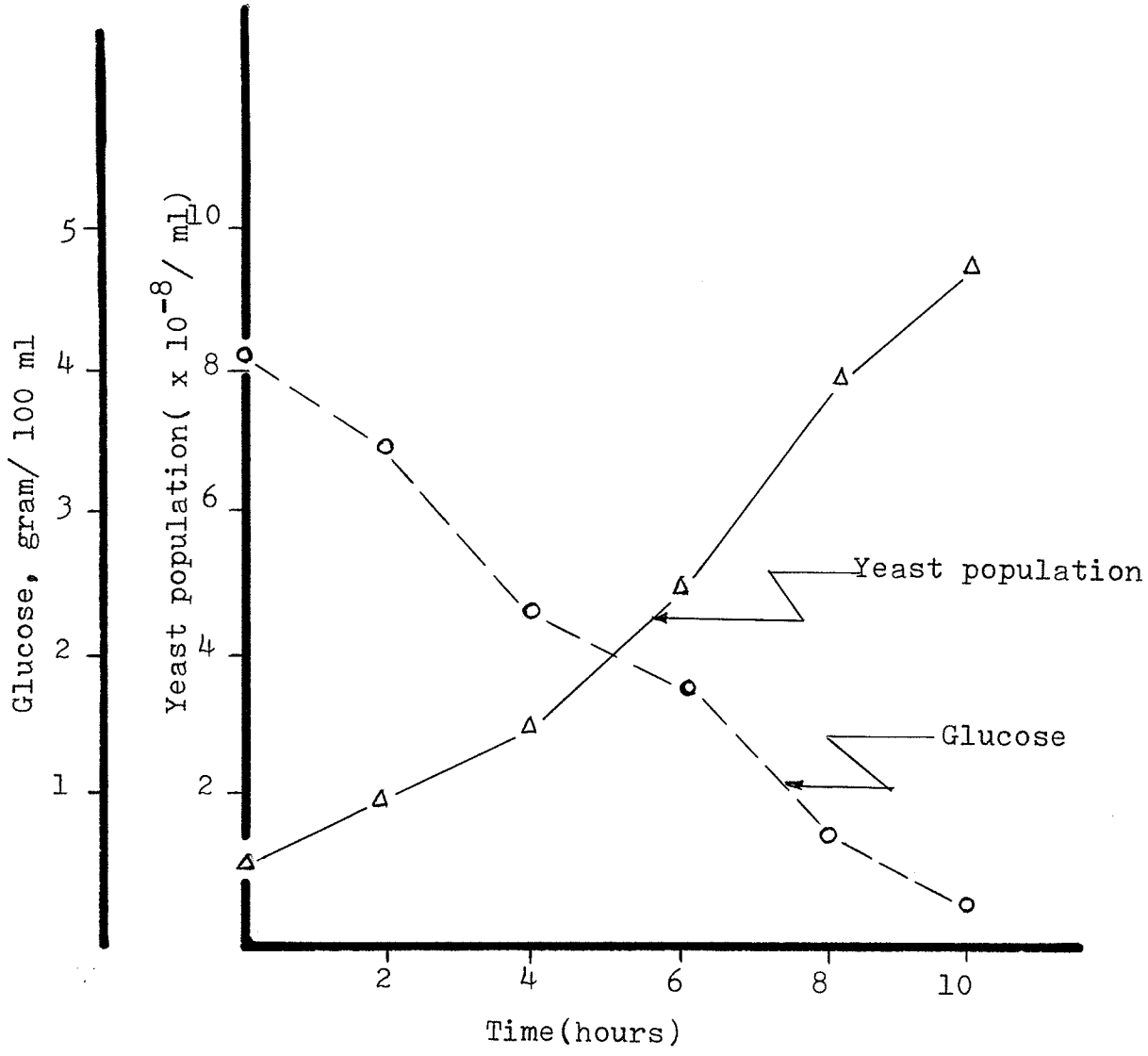


Figure 2.2 The growth of yeast on HCl-hydrolyzate of potato starch

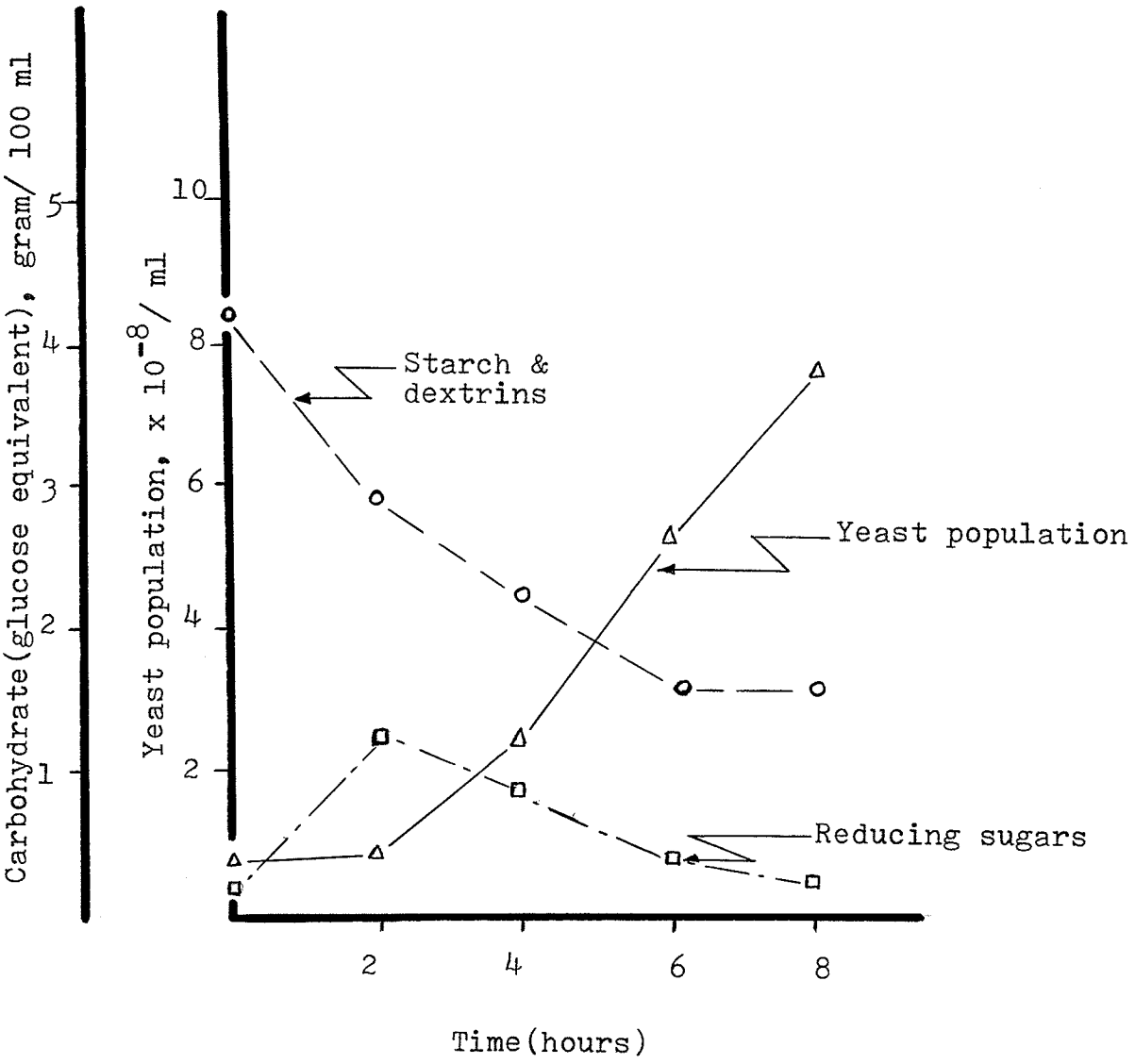


Figure 2.3 The growth of yeast and the change in starch content of the medium during symbiotic growth of E. fibuliger and C. utilis

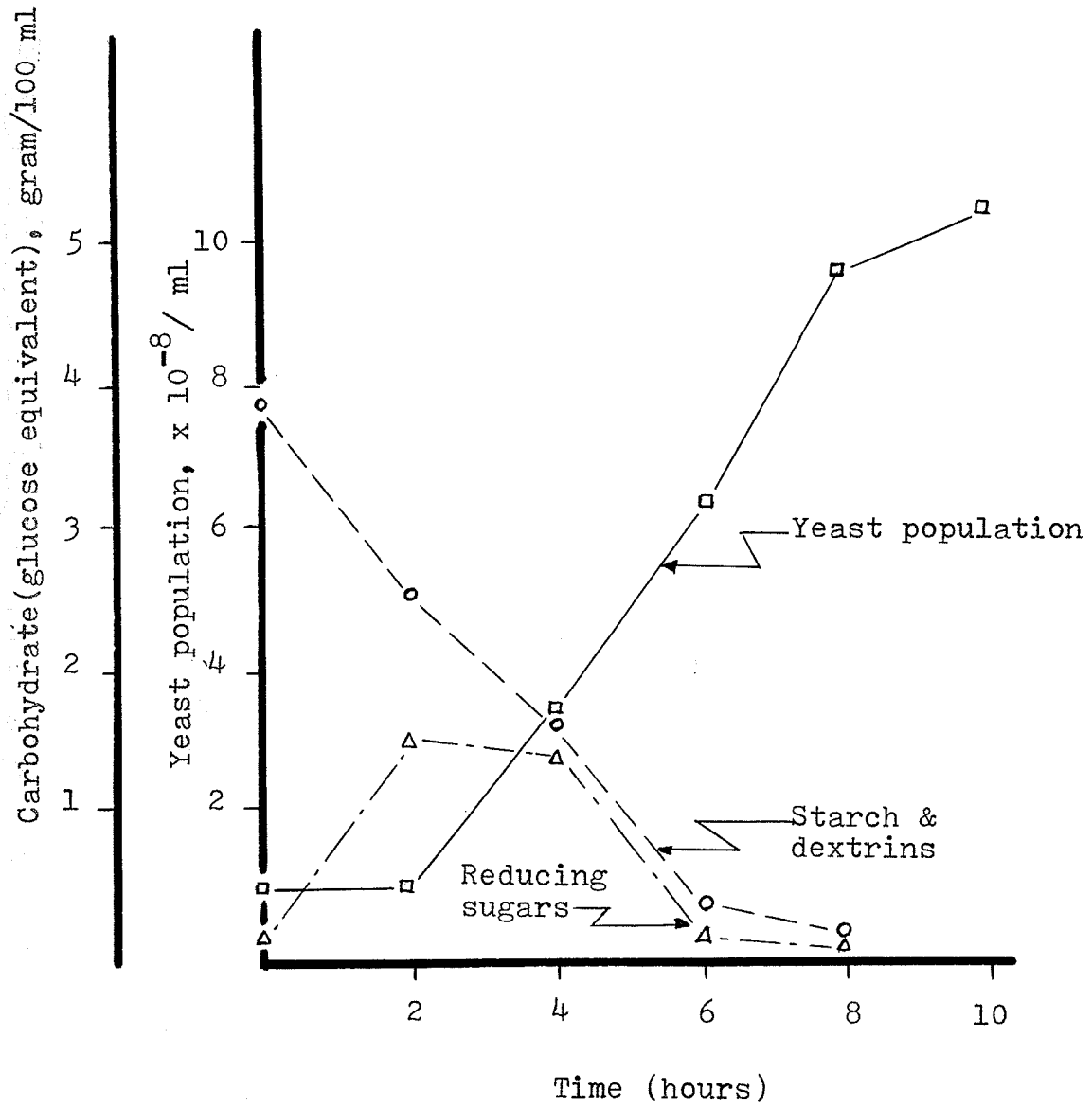


Figure 2.4 The growth of yeast and the change in starch content of the medium during symbiotic growth of E. fibuliger, A. awamori and C. utilis

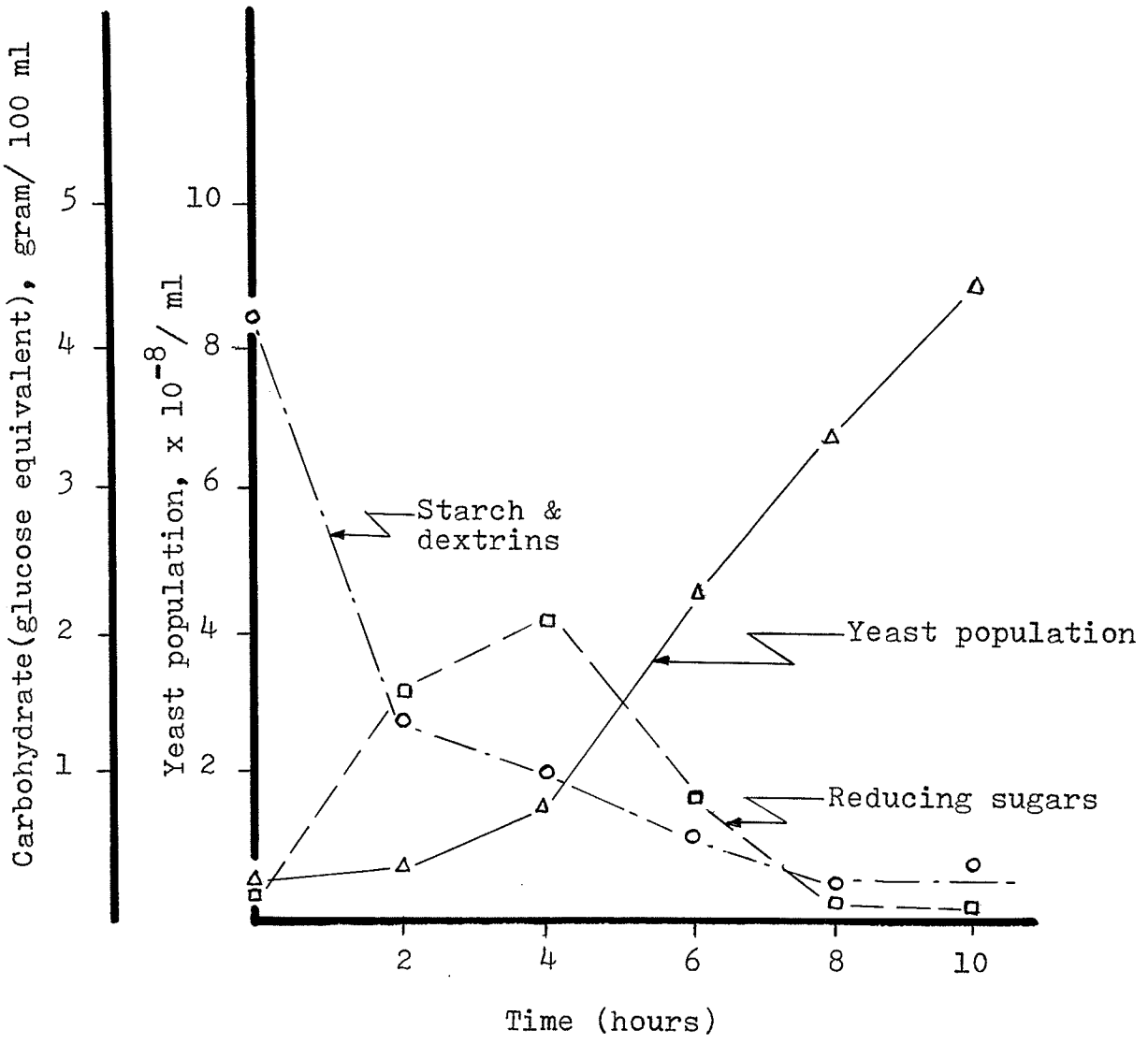


Figure 2.5 The growth of yeast and the change in starch content of the medium during symbiotic growth of E. fibuliger, C. utilis and A. niger

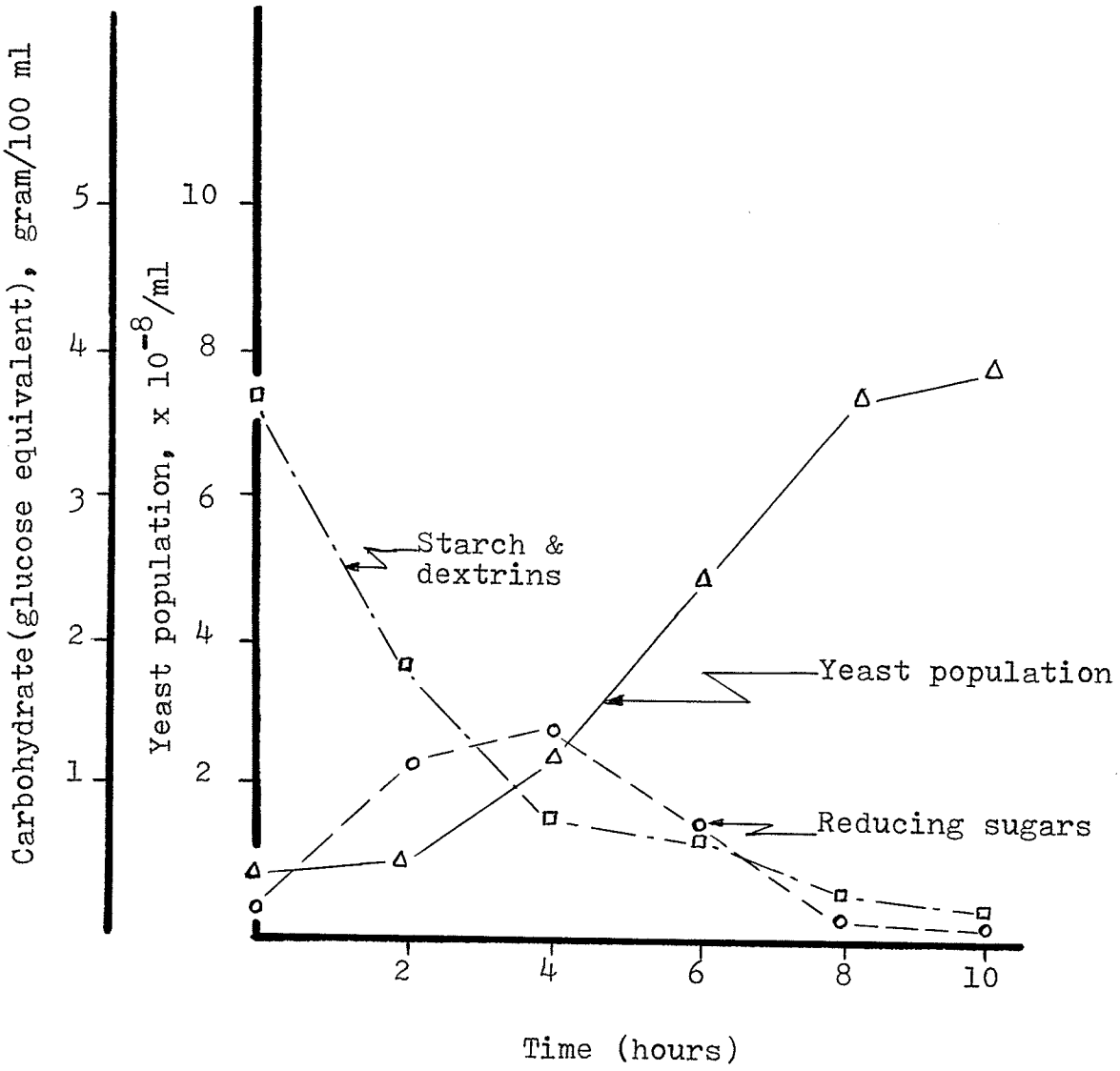


Figure 2.6 The growth of yeast and the change in starch content of the medium during symbiotic growth of A. niger and C. utilis

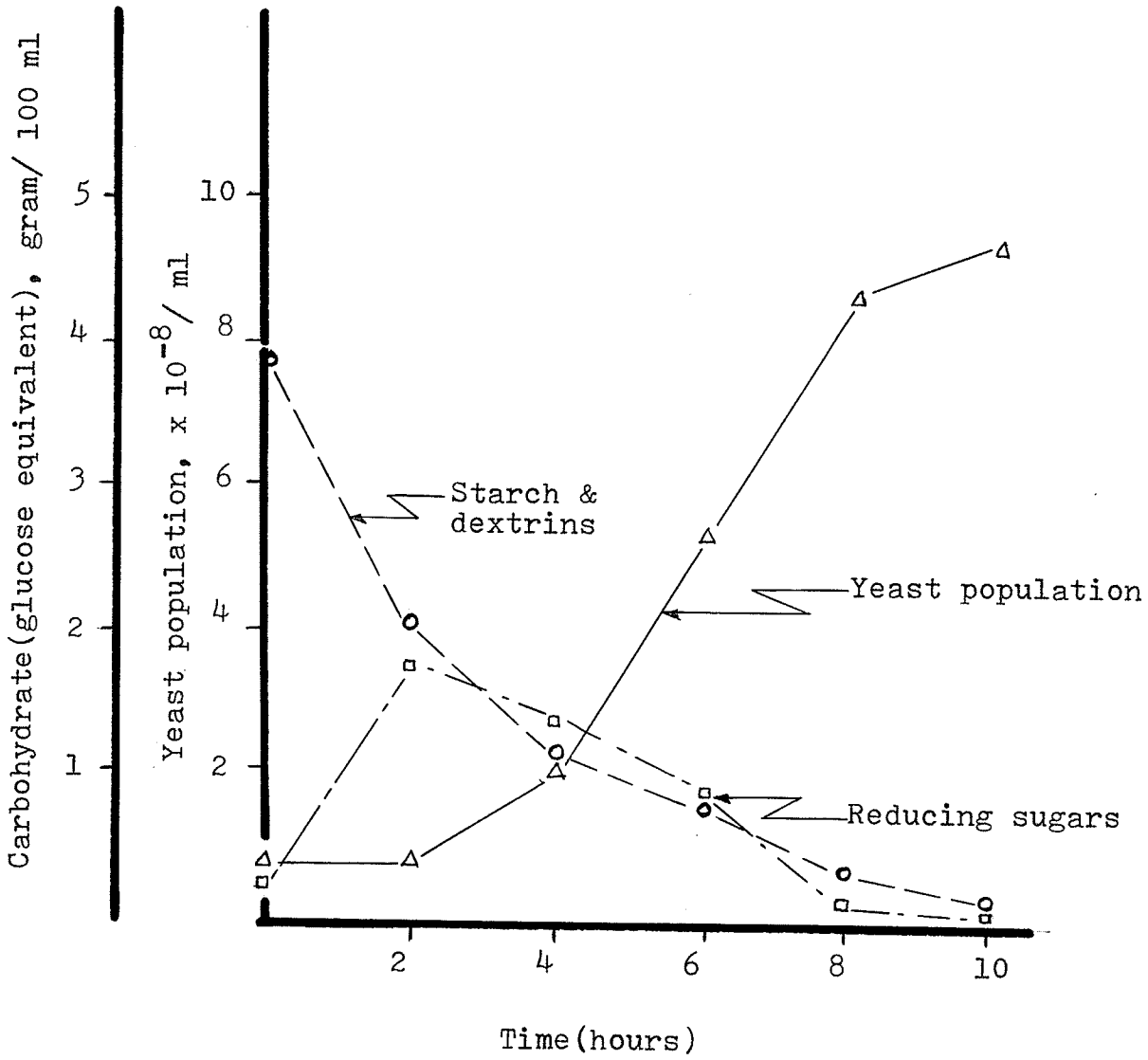


Figure 2.7 The growth of yeast and the change in starch content of the medium during the symbiotic growth of A. awamori and C. utilis

Table 2.7. Comparison of fermentations involving the symbiotic growth of *C. utilis* with different amylase-producing microorganisms

| Organisms Tested | Trial | Yield%* | Utilization of carbohydrate % | Protein content of mixed product % |
|---|---|---|---|---|
| *A. awamori* & *C. utilis* | 1 | 74.9 | 89.2 | 42.5 |
| | 2 | 78.2 | 94.0 | 38.3 |
| | 3 | 69.5 | 90.3 | 39.1 |
| | average | 74.2 | 91.1 | 40.0 |
| *E. fibuliger* & *C. utilis* | 1 | 78.7 | 58.9 | 33 |
| | 2 | 80.2 | 60.9 | 32.6 |
| | average | 79.5 | 59.9 | 32.8 |
| *C. utilis* grown on potato HCl-hydrolyzate | 1 | 61.8 | 91.2 | 44.1 |
| | 2 | 57.5 | 92 | 45.3 |
| | average | 59.7 | 91.6 | 44.7 |
| *A. niger* & *C. utilis* | 1 | 76.8 | 90.5 | 35.6 |
| | 2 | 69.3 | 92.4 | 37.5 |
| | 3 | 72.6 | 92.8 | 36.8 |
| | average | 72.9 | 91.9 | 36.6 |
| *A. awamori*, *E. fibuliger* & *C. utilis* | 1 | 78.9 | 93.5 | 39.8 |
| | 2 | 75.2 | 90.5 | 40.5 |
| | 3 | 74.2 | 95.9 | 40.2 |
| | 4 | 75 | 93.9 | 39.9 |
| | 5 | 76.2 | 91.8 | 42.4 |
| | average | 75.9 | 93.1 | 40.6 |
| *A. niger* *E. fibuliger* & *C. utilis* | 1 | 68.5 | 92.1 | 39.8 |
| | 2 | 74.1 | 89.9 | 33.4 |
| | 3 | 78.2 | 85.1 | 37.3 |
| | average | 73.6 | 89 | 36.8 |

* Based on the initial starch content.

to be dependent on the initial starch content and the activity of the starch-hydrolyzing organisms. For a 90% reduction of starch content of the mash, the fermentation time was found to be about 8 to 10 hours.

This study is believed to be the first in which amylase synthesizing microorganisms have been intentionally used for the production of a nutritional product. Kuehner and Wickerham (79), and Jarl (84) have studied the symbiotic growth of microorganisms with starchy materials as substrate, but their work was apparently confined to the symbiotic growth of C. utilis and E. fibuliger. As was mentioned before, our investigation was aimed at the conversion of starch into yeast cells by growing C. utilis in symbiosis with starch-hydrolyzing organisms. The yield, based on starch consumed, and crude protein content were of concern to us. Wickerham and Kuehner (86), and Tveit (81) patented their respective processes of growing C. utilis to obtain a yeast product of about 35 to 40% protein and 60% yield (based on starch consumed by a symbiotic process). The detailed data of fermentation, however, were not given. It is generally believed that E. fibuliger produces extracellular amylolytic enzyme systems, so that $\alpha$- and $\beta$-limit dextrins should be formed in the fermentation mash. C. utilis is known to utilize only glucose and maltose but not $\alpha$- or $\beta$-limit dextrins released by E. fibuliger, thus the yield of yeast and decrease in starch would not reach the maximum. The lower protein content of the yeast product and the lower

carbohydrate utilization obtained in Wickerham's or Tveit's work or this study, from a symbiotic propagation of C. utilis and E. fibuliger indicates that this hypothesis may be correct.

A. awamori is a microorganism that contains a very powerful glucoamylase system. Glucoamylase hydrolyses not only amylopectin and the amylose of starch but also various limit dextrins to glucose. This is the primary reason why A. awamori was introduced in the symbiotic growth of C. utilis and E. fibuliger. The Aspergilli amylase system has been extensively studied, and found to have an optimum pH value of 4.0 to 6.0 (64, 69, 70, 72, 73, 74, 75, and 76). In addition, Aspergilli and Endomycopsis amylases both are found to have optimum temperature of 40 to 50°C. C. utilis, on the other hand, is found to be grown best at pH 3.5 to 5.0 with optimum temperature of 35°C. The fermentation conditions of our study were, therefore, chosen to be pH 4.5 and 35°C. When one compares Kuehner and Wickerham's (79) and Tveit's and Jarl's (84) results, it could be concluded that the results, 75.9% yield of dried solid and 40.6% crude protein, from this study by a symbiotic propagation of E. fibuliger, A. awamori and C. utilis in potato starch represent a significant improvement in protein content of the product.

By comparing the results presented in Table 2.7, it could be concluded that the symbiotic growth of C. utilis, A. awamori and E. fibuliger gave the most protein synthesis, highest utilization of carbohydrate, and maximum yield. C. utilis

grown in HCl-hydrolyzate, gave a high protein product, but the yield was low. The symbiotic propagation of C. utilis and E. fibuliger gave the lowest protein synthesis and carbohydrate decrease. Although A. niger has been shown to be an organism that produces much glucoamylase, it was less effective than A. awamori in these symbiotic cultures. The protein synthesized from either the symbiotic growth of A. niger and C. utilis or of E. fibuliger, A. niger and C. utilis was not as great as that from the mixed culture of C. utilis, E. fibuliger and A. awamori.

Cadmus et al (70) studied the enzymatic production of glucose syrup from grains. Starch from gound samples of whole corn, wheat, and sorghum was enzymatically converted to glucose in 90-99% yield with a combination of $\alpha$-amylase from either barley malt or Bacillus, and glucoamylase from Aspergillus species. Barley malt or Bacillus was necessarily added to produce $\alpha$-amylase in order to reduce the viscosity of the starchy mash. In the present study, E. fibuliger, one of the $\alpha$-amylase-producing microorganisms, produced $\alpha$-amylase in the fermentation mash. Thus no barley malt or Bacillus was necessary for the reduction of viscosity during the symbiotic culture of yeast on the starchy mash. It could be concluded that E. fibuliger will reduce the initial viscosity of the mash making it easier to handle in pumps and other equipment usually found in a yeast factory.

The carbohydrate turn-over during a batch operation of

these symbiotic fermentations (Figs. 2.3 to 2.7) revealed that the saccharifying activity is the limiting factor. Starch is converted into dextrins and oligosaccharides. Glucose and maltose are thought to be assimilated by C. utilis as soon as they are formed. However, reducing sugars were found to accumulate during the first 6 hours when the growth of C. utilis was not in the logarithmic phase. As the growth of C. utilis went into the logarithmic phase, it utilized most of the reducing sugar that was present. At the final stage of growth, reducing sugars were found in quite low concentration. Since Endomycopsis excretes $\alpha$- and possibly $\beta$-amylase and A. awamori excretes glucoamylase, it is possible that there are different oligosaccharides formed during the symbiotic fermentation. An attempt should be made to identify these oligosaccharides by paper chromatography or gas chromatography.

It is evident from the data that the efficiencies of protein production, on the basis of starch supplied or starch utilized, were quite low for all microorganisms tested. The protein content of the dried product was found to be around 40% and was lower than commercial yeast products which contain about 50% crude protein. In the present study, since unpeeled potato tubers were used as the only carbon source, potato peelings and fibers (epidermis, cell walls) would remain in the mash and be mixed with the yeast cells after centrifugation, thus leading to lowering of the protein content of the dried yeast product. Such

a situation cannot easily be imporved when potato waste is employed as the carbohydrate source for C. utilis multiplication. However, such a product can be used as a feed supplement since the major part of the mixed product is C. utilis cell substances and in most respects it is comparable in quality to ordinary commercial Torula yeast.

Industrial application of amylolytic microorganisms for the production of nutritive materials from starch-containing substrates could assume two forms: (1) a process in which a portion of a starch-containing crop, such as culled potatoes, is used for the production of food yeast, and (2) a process in which a starch-containing waste is utilized. In the first instance a mash concentration as high as could efficiently be handled would be desirable in order to make the most efficient use of the capital investment. In the second process, the utilization of large amounts of dilute materials, often containing as little as 1 per cent, or less, of solids would be required. Whether it would be desirable to operate at these lower solids concentrations would depend upon the costs of operation with such dilute materials, as well as the demand for yeast, and the efficiency of the process in reducing the biological oxygen demand. It should be pointed out, however, that yeast fermentation would not be considered a full solution to the possible pollution problems, since the BOD of the industrial wastes likely to be used for yeast production would only be reduced by a little over 50%

according to Kuehner (78). Further experiments are needed in regard to the fermentation of waste materials by yeast, before the value of the process can be fully established.

## Section II. Fermentation of Potato Starch by a Continuous and Symbiotic Process


### Abstract

A product of high protein content was obtained by continuous propagation of mash made from whole potatoes with a mixed culture consisting of E. fibuliger, A. awamori and C. utilis. The mash was composed of potato starch, urea, ammonium sulfate, and potassium phosphate, and was adjusted to pH 4.5 before propagation. Continuous fermentation was initiated when the yeast population of the fermented mash reached approximately 1 x $10^9$ cells per ml. Fermentation was carried out at pH 4.5 and at 35°C. with vigorous aeration and agitation. The exchange rate during continuous fermentation was approximately 15-17% (v/v) per hour. A product containing 38-42% crude protein was obtained at 73% yield based on the average initial concentration of starch.


### Introduction

From the industrial point of view, the continuous cultivation of yeast will offer obvious technical and economic advantages. A continuous process is generally based on the growth conditions for batch fermentations, and is initiated by growing at batch culture to a suitable cell density. A continuous flow of fresh, sterile substrate is then fed into the cultivation vessel and an equivalent volume flows out so that the operating volume remains constant. To keep the cell density at a desirable level, the rate of cell production has to equal the rate of washout

(output). Hence, the dilution rate at steady state will be equal to the growth rate constant. In continuous cultivation, a constant high rate of biomass formation prevails.

Continuous propagation of yeast has been used commercially with sulfite liquor, wood hydrolyzate, and molasses as raw material. Harris et al (22) carried out a laboratory scale continuous fermentation of sulfite liquor with C. utilis. Jarl (84) and his colleagues have thoroughly studied a symbiotic system on a laboratory scale, using a mixed culture of E. fibuliger and C. utilis. The process has been scaled up to pilot scale; the equipment includes 5 fermentors (up to 1,000 liter volume) and is designed for a maximum flow rate of 200 1/hr. corresponding to a production of about 100 kg yeast per day.

The present study was undertaken to extend the batch process of symbiotic growth of A. awamori, E. fibuliger and C. utilis to a continuous and symbiotic phase. Attempts were made to obtain better conditions for protein synthesis and carbohydrate reduction during such a symbiotic process. Much attention was paid to developing a process suitable for commercial adaptation.

## Material and methods

Whole potatoes were used throughout this study. Unpeeled potato tubers were washed in cold tap water and throughly ground in a Waring Blendor.

In preparation of the medium for batch fermentations,

the ground potatoes were brought to approximately 4% starch concentration with tap water, and nutrients (0.05% $K_2HPO_4$, 0.25% urea and 0.25% ammonium sulfate) were added to the potato mash before its pH was adjusted to 4.5. The potato mash was then sterilized by autoclaving for 1 hour at 248°F. and employed as the fermentation mash.

Two procedures were employed for the preparation of the mash for continuous fermentation. In the first procedure, mash was prepared without pretreatment with malt. Ground potato mash was autoclaved at 248°F. for 1 hour and then kept in a refrigerator at 4°C. Before use, this sterilized potato mash was adjusted to approximately 4% starch concentration with tap water. Nutrients (0.05% $K_2HPO_4$, 0.25% urea and 0.25% ammonium sulfate) were added to the 4% potato mash.

In the second procedure, approximately 8 pounds of ground potato mash was mixed with 1.5 liters tap water and 30 g ground malt. The mixed potato mash was autoclaved at 248°F. for 1.5 hours and then cooled to 55°C. One liter of E. fibuliger and A. awamori (20:80) was added to the sterilized potato mash and the mixture kept in an incubator at 55°C. for 2 hours. This partially converted potato mash was then used to prepare the continuous fermentation mash as described in the first procedure.

All continuous runs were performed in a modified Waldhof fermentor built in our laboratory (Fig. 2.1). The pH of the mash was controlled automatically at 4.5 by a pH-auto-

controller. Eight liters of sterile medium described previously was inoculated simultaneously with 10% of E. fibuliger and A. awamori (20:80), and a culture of C. utilis to bring the yeast cell population of the mash to 1 x $10^8$/ml. A suitable concentration of yeast cells was established by culturing the mash at 35°C. while aerating with 1 liter of air per liter of mash per minute. The flow of medium for continuous fermentation was started when the yeast cell population of the fermenting mash reached approximately 1 x $10^9$ cells per ml (usually about 10 hours after inoculation).

The composition of the continuous medium was the same as that of the initial fermentation mash described previously, except that the starch content and nutrients varied according to the fermentation conditions. The most often used continuous media were obtained by mixing amylase-treated sterile starch mash with the nutrients (0.05% $K_2HPO_4$, 0.25% urea and 0.25% ammonium sulfate). The pH of this mash was not adjusted before pumping the mash into the fermentor.

Two metering pumps were used in the continuous fermentation system. The medium used in the continuous fermentation was pumped into the fermentor by a metering pump. The ripened medium was pumped out from the fermentor by another metering pump. The rate of exchange of continuous medium and fermented mash was dependent on the number of yeast cells in the fermented mash. Attention was paid mainly to the growth of C. utilis and the

changes in carbohydrate content of the medium during fermentation.

Samples for carbohydrate, protein and dry matter determinations were taken every 4 hours. Each sample was mixed well and centrifuged at 3000 r.p.m. for 10 minutes. The precipitate of each sample was dried in an oven at 105°C. for 16 hours and calculated as percentage dried solids. The supernatant was sampled for residual carbohydrate and sugar analysis.

The methods used for the determination of reducing sugars, protein and yeast cell population were the same as those described in Section I.

## Results and Discussion

### A. Growth of yeast in a non-amylase-treated mash

Data on yeast growth in a symbiotic and continuous culture of A. awamori, E. fibuliger and C. utilis in a non-amylase-treated mash are presented in Fig. 3.1. The average initial starch content of the continuous mash was 4836 mg per 100 ml and the average residual carbohydrate was 1049 mg/100 ml. The utilization of carbohydrate was calculated to be 78.3%. The dried solid from the fermented mash was 3.4 g/100 ml of mash with a protein content of 38.2%. The average exchange rate was 1.17 l per hour per 8 l culture, or approximately 15% per hour.

The possibility of increasing the activity of amylase during the continuous and symbiotic process was investigated as

only very small quantities of amylase were present in the fermented mash. It was found that a mixed culture of E. fibuliger and A. awamori could be added to the fermenting mash to compensate for the loss of amylase activity during the continuous fermentation. Amylase activity which produced 20 mg dextrose per ml enzyme solution per hour at 60°C. was found to be enough to carry out the process. If the enzymic activity was lower than this level, the yeast population decreased, but would increase again soon after the enzymic activity was increased. Since enzyme formation is a time consuming process, the amount of amylases formed from E. fibuliger and A. awamori may not be sufficient to meet the requirement for amylases during the symbiotic and continuous process. A study of means to speed up the formation of amylases during the symbiotic and continuous process is suggested for the future.

Harris et al (22) conducted a four-month continuous fermentation with C. utilis with sulfite liquor as a carbohydrate source but without using aseptic conditions. No contamination was observed as long as the pH was held at 5.0. However, if the pH rose to 6.0, bacterial contamination became evident. This contamination disappeared as soon as the pH was readjusted to 5.0. In the present study, contamination was evident although the pH was kept at 4.5. The major reason for contamination was that the sterile starch mash was invaded by molds, even when the mash was kept in the refrigerator under sanitary conditions. The vessel used to hold the continuous medium was contaminated after

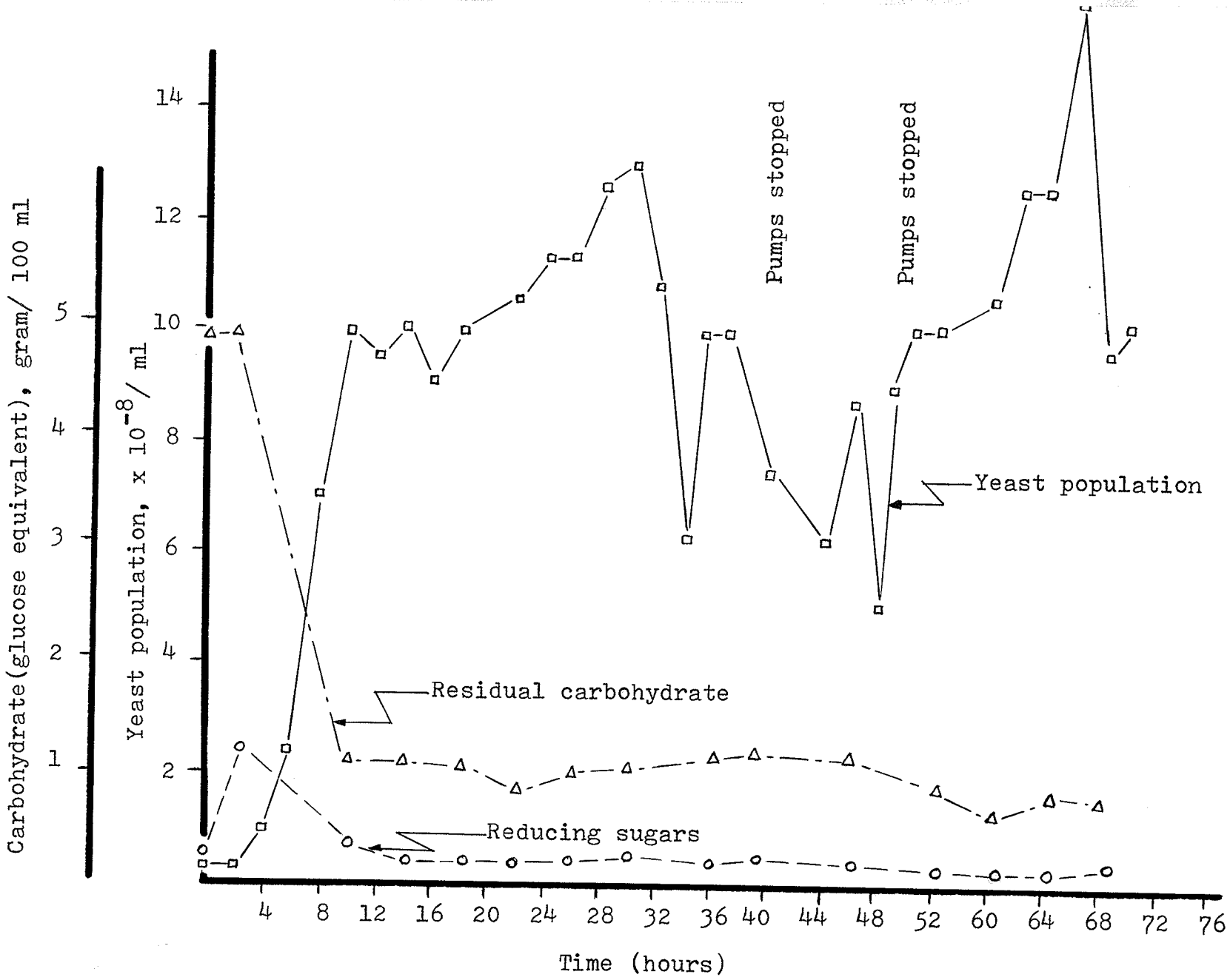


Figure 3.1 The growth of yeast and the change in starch content of the medium with a non-amylase-treated potato mash as carbon source for a symbiotic and continuous culture of E. fibuliger A. awamori and C. utilis

one day's continuous performance without washing. After resterilizing the continuous medium and washing all the equipment, the contamination was reduced but not entirely eliminated.

In this study, a continuous period of 68 hours, including eight hours of establishing the desired yeast concentration, was achieved. Theoretically, if no contamination problem or no yeast activity problem occurs in the continuous mash, and if the nutrients of the continuous mash are suitable for multiplication of the organisms, the continuation period should be unlimited. Because of the complications of the symbiotic system, the duration of the continuous process may be somewhat limited. Further tests are required to ascertain the period for which a continuous symbiotic culture can be maintained.

The result of the continuous process in comparison with that of the batch process was not very significant. The comparative results of the batch and the continuous yeast propagation processes are summarized as follows:

| | Process | |
|---|---|---|
| | Batch | Continuous |
| Utilization of carbohydrate during propagation (glucose equivalent) | 93.1% | 78.3% |
| Percentage yield of final product, based on starch consumed | 75.9% | 71.1% |
| Crude protein content, approx. | 40.6% | 38.2% |
| Residual carbohydrate, mg/100 ml | 108 | 1049 |

The poor result of this study could be due to the exchange

rate used, although it was only 15% (V/V) per hour. Generally speaking, amylases require several days to convert starch to sugars, so complete conversion could not be expected in the 6 to 8 hours allowed by this exchange rate. Conceivably, it could also be thought that C. utilis was in partial starvation while this process was being performed. The residual carbohydrate content of the medium was high in this study when compared to the batch process. Since the utilization of carbohydrate was 78.3% and crude protein content of the final yeast product was 38.2%, the results of this study would not attract much attention from industrial sources.

The exchange rate for this continuous run was approximately 1.17 l per hour. The daily production of fermented mash was 28 liters. If yeast fermentation is carried out by a batch process, there will be 8 liters of fermented mash produced daily from a 17 liter capacity fermentor. In comparison, if continuous propagation is performed, at least 28 liters of fermented mash, according to this study, will be harvested. The following comparison might be used to give a clearer picture.

| Propagation method | Daily fermented mash collected | Daily final yeast product harvested |
|---|---|---|
| Batch | 8 l | 280 g |
| Continuous | 28 l | 980 g |

In conclusion, the productive capacity of the yeast fermentor will be increased 3.5 times if a continuous process is employed.

B. The growth of yeast in an amylase-treated mash

Data on yeast growth in a symbiotic and continuous culture of A. awamori, E. fibuliger and C. utilis with an amylase-treated mash are presented in Fig. 3.2. The average initial starch content of the continuous mash was 4140 mg/100 ml and average residual carbohydrate content was 392 mg per 100 ml. The utilization of carbohydrate was calculated as 90.4% and the yield was 73% based on the average concentration of starch. The exchange rate of the propagated mash was 1.37 l per hour per 8 l of fermenting mash and equalled approximately 17% of the actual volume of the culture.

The major aim in this study was to improve the crude protein content of the product and the simultaneous reduction of carbohydrate content during the symbiotic process. Since the limiting factor in the previous test was probably due to the lack of amylase activity, attention was paid in this study to pre-treating the mash with a mixed culture of E. fibuliger and A. awamori and holding the mash at 55°C. for 2 hours. The data indicate that growth of C. utilis was significantly improved as compared with that in the previous study. A comparison of these results is shown in Table 3.3.

Table 3.3. The comparison of yeast growth in a symbiotic and continuous process, with non-amylase-treated and amylase-treated starch mash as carbohydrate source.

| | Non-amylase-treated | Amylase-treated |
|---|---|---|
| Average carbohydrate content of mash, mg/100 ml | 4836 | 4140 |
| Residual carbohydrate of fermented mash, mg/100 ml | 1049 | 392 |
| Utilization of carbohydrate,% | 78.3 | 90.4 |
| Crude protein content of final product,% | 38.2 | 42.1 |
| Exchange rate, 1/8 1/hr. | 1.17 | 1.37 |
| Exchange rate, % | 14.6 | 17.1 |

All carbohydrates are expressed as glucose equivalents

From these results, it can be concluded that the amylase-treatment of the starch mash brought about a significant increase in protein synthesis and better utilization of carbohydrate during fermentation. The utilization of carbohydrate in this study was 90.4%, nearly equivalent to the carbohydrate utilization in a batch process. The protein content of the final mixed product was 42.1%, higher than the average protein content (40.6%) of the batch process. The residual carbohydrate was 392 mg/100 ml, a tremendous improvement over the residual carbohydrate level, 1049 mg/100 ml, from the previous study. It is evident that the amylases hydrolyzed more starch during the pre-treatment with the mixed culture of E. fibuliger and A. awamori.

With respect to the contamination problem, no contamination was found in this study. A 67-hour period of continuous

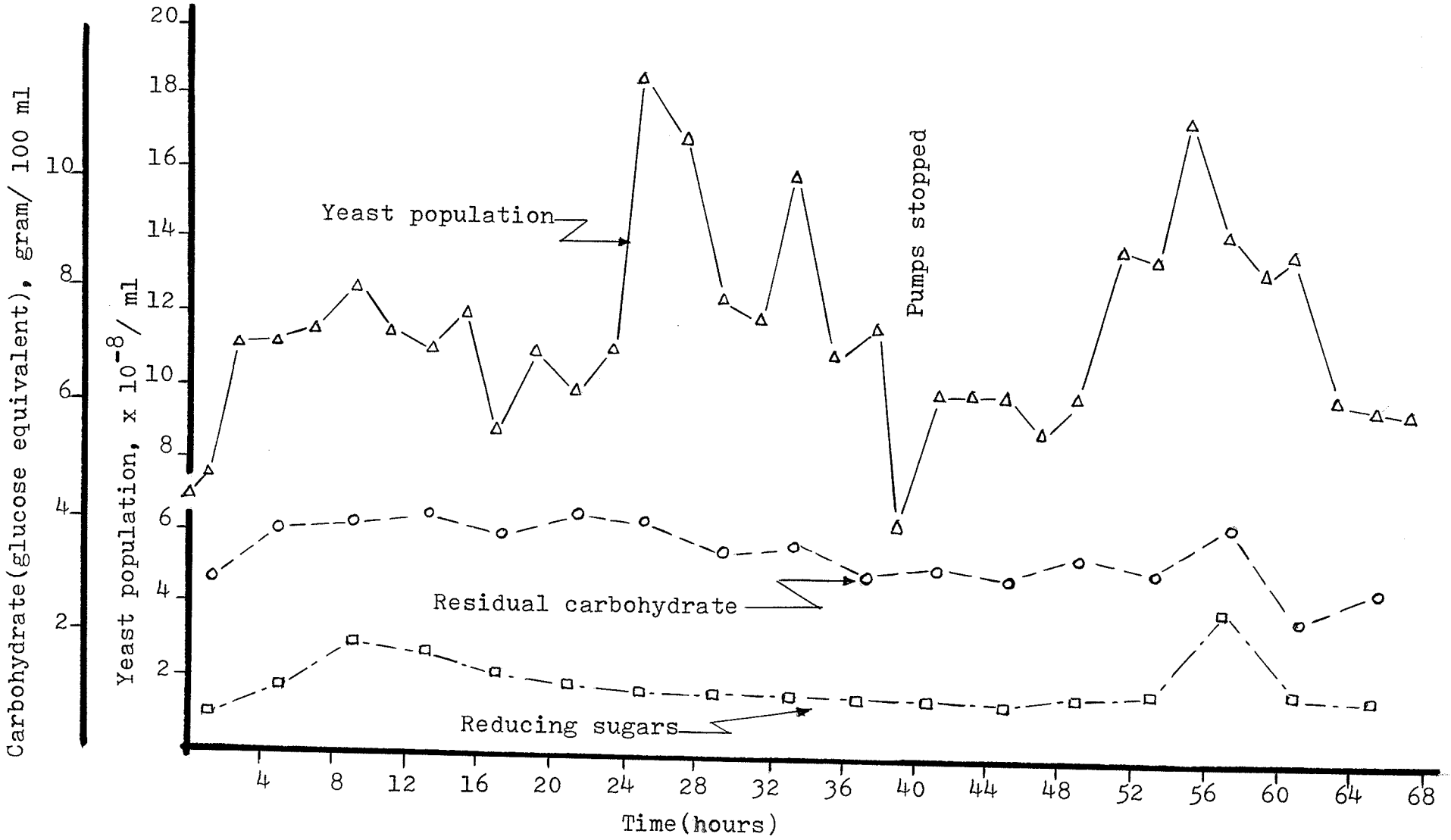


Figure 3.2 The growth of yeast and the change in starch content of the medium, with an amylase-treated potato mash as carbon source for a symbiotic and continuous culture of E. fibuliger, A. awamori and C. utilis(the 10 hr. start-up period is not shown)

fermentation was achieved in this study, although it could not be compared with the work of Harris (22) of a four-month continuous fermentation. Since the pH of the fermentation mash was kept at 4.5 and the feed mash was sterilized, it might be expected that the length of continuous propagation could be as long as Harris'.

The exchange rate obtained from this study was 17% of fermented mash volume per hour, and can be considered as an improvement. Around the world, the exchange rate of fermenting mash of sulfite liquor, molasses and wood hydrolyzate is approximately 20% on an industrial scale. The exchange rate obtained from this study, therefore, could be thought to be competitive with that which is currently being achieved in industry.

Since the mash had received an amylase-treatment, it can be assumed that more vessels will be required, for the industrial application of this process. However, the higher protein content of the final yeast product, and greater utilization of the carbohydrate, could overcome the disadvantages of a higher investment in equipment.

A fluctuation of yeast cell population was found in both studies. When no contamination problem occurred during the process, the fluctuation of yeast cell population might be caused by the uneven exchange rate due to occasional pumping problem, that kept the yeast population in an unsteady state. When contamination occurred in the continuous system, the growth of C. utilis would be reduced eventually. Unless certain suitable assistance is given

to the propagation of C. utilis to help its activity, it could not compete with contaminating organisms even if the pH of the fermentation mash is held at 4.5. Furthermore, this study was conducted with the aid of four graduate students on duty at irregular times. One possible factor of the measured fluctuation, could be attributed to the person-to-person error in estimating yeast cell populations, using the Levy Count Chamber.

## Section III. Changes in Carbohydrates During the Fermentation of Potato Mash by a Symbiotic Process


### Abstract

The changes in carbohydrates during the growth of yeast in a symbiotic culture with potato starch as substrate was investigated. Ascending paper chromatography was conducted to identify the changes every two hours during the growth period. Glucose and dextrin were found to be the major end products due to the amylase action of E. fibuliger. No strong evidence was found to suggest that E. fibuliger excretes both α- and β-amylases during its growth. Glucose and dextrin were also found to be present in the mash during the symbiotic growth of A. awamori or A. niger and C. utilis. Maltose was formed as an intermediate of starch conversion and then converted to glucose. Evidence, obtained from paper chromatography, showed that α-amylase was excreted by E. fibuliger, A. awamori and A. niger during their growth in symbiosis with C. utilis.


### Introduction

Two important types of industrial enzymes, endo- and exo-amylases, are produced by microorganisms to hydrolyze the α-1,4 bond of starch and related substrates to yield hydrolyzates of commercial value. Endo-amylases are α-amylases which rapidly dextrinize large polymers by cleaving internal bonds to yield a heterogenous product. Exo-amylases, on the other hand, are β-amylase and glucoamylase (r-amylase) which attack the non-reducing

end of the substrate molecule, producing maltose and glucose respectively. Beta-amylase cannot bypass 1,6 linkages in a starch chain while the glucoamylase is capable of hydrolyzing $\alpha$-1,6 as well as the $\alpha$-1,4 linkage and can convert starch completely to glucose.

Alpha and glucoamylases are widely distributed in many species of microorganisms, and in higher plants and animals, but $\beta$-amylase has previously been found only in higher plants. Recently, it was also found that E. fibuliger produces both $\alpha$-amylase and $\beta$-amylase (48, 49, 50, 51).

Since a mixed culture of organisms was employed to synthesize yeast protein from potato mash in this study, a complicated starch-hydrolyzing enzyme mixture probably developed. Conceivably this enzyme system could demonstrate a different amylase activity to that which occurs in the growth of single organisms alone. In this investigation, major efforts were aimed at studying the development of amylase activities during symbiotic growth, by using paper chromatography techniques for the identification of carbohydrate changes. No attempt was made for the purification or kinetic study of amylases.

Microorganisms tested

Aspergillus niger NRRL-3122 and A. awamori NRRL-3122 were used for the production of glucoamylase. Endomycopsis fibuliger NRRL-Y-25 was used for the production of $\alpha$-and, perhaps, $\beta$-amylase. C. utilis NRRL-900 was used to synthesize protein

from the carbohydrates converted by A. niger, A. awamori or E. fibuliger.

C. utilis was kept on malt extract-yeast extract (MY) agar containing 3 g malt extract, 3 g Difco yeast extract, 5 g peptone, 10 g glucose, 20 g agar and one liter of water. The pH, which was not adjusted, varied between 5 and 6, depending upon the particular batch of ingredients. The amylase-synthesizing organisms, E. fibuliger, A. niger, and A. awamori, were maintained on slants of a soluble starch-malt extract-yeast extract agar. The composition of this medium was the same as that of MY agar except that 10 g of soluble starch were used in place of glucose (79).

Materials and methods

Since this study was initiated with the purpose of meeting the requirements of industry, every attempt was made to simplify the procedure so that it could be directly used by industry at a low cost. Only potato starch was used as the carbohydrate source for all microorganisms. Whole potato and/or potato peels were ground into a fine state in a Waring Blendor, standardized to approximately 4% starch, and sterilized in an autoclave at 248°F. for 30 minutes. Prior to the introduction of the test organisms into the mash, urea, 0.25%; ammonium sulfate, 0.25%; $K_2HPO_4$, 0.05% were added to the medium. This mash was used to initiate the growth of C. utilis in the fermentor during the symbiotic process for the purpose of increasing the population of yeast cells.

The mash used for the production of amylase by Aspergilli and Endomycopsis was prepared as described for C. utilis propagation, except that 0.25% of Difco yeast extract was used in place of the salts. A 24-hour-old culture was used as the primary inoculum in all experiments.

Inoculum of the starch-hydrolyzing organisms, namely A. niger, A. awamori and E. fibuliger, was prepared by inoculating 50-ml portions of sterile mash, containing 4% starch and 0.1% Difco yeast extract without pH adjustment, with a loop of microbial cells from the agar slant. Flasks so inoculated were placed on a Gallenkamp reciprocal shaker operating at 200 r.p.m. with a 2-inch stroke. These cultures were incubated on the shaker for 2 days at 28°C. for E. fibuliger, and 35°C. for A. niger or A. awamori. At the end of this period, these cultures were used to inoculate the fermentors.

The C. utilis inoculum was cultured on a medium containing 3% glucose, 0.15% $K_2HPO_4$, 0.05% $MgSO_4.7H_2O$, 0.2% urea and 0.01% Difco yeast extract, at pH 4.5. 50 ml of this medium was placed in an Erlenmeyer flask and autoclaved at 248°F. for 15 minutes. Each flask of medium was inoculated with a loopful of microbial cells from the agar slant. Cultures were incubated for 24 hours on the Gallenkamp shaker at 35°C. The cells centrifuged from this culture were used to inoculate the fermentor.

The culture of yeast by the symbiotic process was

performed in a modified Waldhof fermentor, which was built in our laboratory. The capacity of the fermentor is 17.2 liters. Eight liters of medium were usually used in each batch. All fermentations were initiated by inoculating the mash with 10% of 48-hour-old cultures of A. niger, E. fibuliger and A. awamori. The ratio of E. fibuliger to A. niger or E. fibuliger to A. awamori was maintained at 20:80. C. utilis was added at a rate such that the concentration of yeast cells in the medium was $1 \times 10^8$ per ml. During the period of growth, the mash was maintained at a temperature of 35°C., pH 4.5 and the entire system was vigorously aerated and agitated. After about 10 hours, there generally was no more increase in yeast population, and the batch tests were terminated.

After the initiation of symbiotic growth, samples were taken every two hours and 50 ml of fermented mash was centrifuged at 3000 r.p.m. for 15 minutes. An aliquot of supernatant was dialyzed against 4 1 of cold & running 0.01 M calcium acetate at 4°C. for 48 hours with two or three changes of the acetate solution. The small amount of precipitate formed during dialysis was removed by centrifugation at 17,000 g for 10 minutes. Loss of enzymic activity was negligible after three months if the supernatant was stored under toluene at 4°C. The enzymic activity of the supernatant was eliminated by heating the supernatant to 100°C. and then cooled to 4°C. The enzyme-free supernatant was then employed for paper chromatography.

The conversion of carbohydrates to sugars by the enzymic reaction of A. awamori, A. niger or E. fibuliger followed the procedure described by Mills Laboratories (87). The reaction products were used for paper chromatography.

Paper chromatography of the reaction products and the enzyme-free supernatants were performed in accordance with the description of Robyt and French (88). Whatman No. 3 paper 14 x 14 inch was washed with distilled water before use. A series of 0.01 ml samples (a platinum loopful) were placed along a line one inch from the bottom of the paper. After the samples had been applied, the paper was shaped into a cylinder and placed in the developing chamber. The solvent system employed was water-ethanol-nitromethane, 23:44:35 parts by volume. For good resolution, two ascents were employed. The chromatograms were developed by rapidly dipping them into a solution containing 1 ml of saturated $AgNO_3$ per 200 ml acetone. The paper was dried and dipped into a solution containing 1 ml of 40% NaOH per 100 ml methanol. Spots developed immediately. The paper was air-dried and rapidly dipped into Kodak F-24 film fixer and immediately placed in water, and the washing was continued for at least 30 minutes before the paper was re-dried.

Results and discussion

Since this investigation was concerned only with the changes that occurred in the carbohydrate fraction of potato wort during symbiotic growth of the microorganisms, attention was

paid mainly to the growth of C. utilis and the change of carbohydrate during fermentation with A. niger, A. awamori or E. fibuliger respectively. Combinations of A. awamori-E. fibuliger-C. utilis and A. niger-E. fibuliger-C. utilis were also studied.

The liquefaction of gelatinized starch by enzymes is usually carried out by the addition of barley malt or Bacillus subtilis culture filtrate. No attempt was made to investigate the α-amylase activity of A. awamori, A. niger or E. fibuliger. The liquefaction of starch, however, was evident immediately after the addition of A. awamori, A. niger or E. fibuliger to the mash. The iodine-starch color test was also performed on the fermenting medium at each sampling. The blue color of iodine-starch mixture turned to light brown as the culture time increased. E. fibuliger showed the best liquefaction effect in comparison with A. niger and A. awamori. Wickerham et al (48) reported that α-amylase activity of E. fibuliger grown on bread and bran culture was much higher than the average α-amylase content of barley. Sánchez-Marroquin (49, 50, 51) found that amylase from E. fibuliger can hydrolyze corn starch to dextrin and a small amount of glucose. The activity was proportional to the substrate concentration within the range of 3 to 10% starch and equalled that of a 10% malt extract. Fukumoto et al (52, 53) compared 84 strains of fungi imperfecti, and found that E. fibuliger was one of the microorganisms which produced liquifying amylase. Hottori and Takeuchi (54) found that E. fibuliger excreted a dextrinogenic amylase and a

saccharogenic amylase. In considering all previous studies, one might conclude that E. fibuliger is a strong α-amylase producing organism especially suitable for symbiotic growth.

Wickerham et al (48), Sánchez-Marroquin (49, 50, 51), Fukumoto et al (52, 53), and Hattori and Takeuchi (54) studied the enzyme induced reactions and all agreed that E. fibuliger is one of the microorganisms which excretes β-amylase, although Wickerham et al (48) doubted their result. In the present study, the crude culture liquid (unpurified enzyme solution) was employed to hydrolyse the starch substrate and maltose. Afterwards this liquid was chromatographed on paper. The result is presented in Fig. 4.1. It appeared that E. fibuliger produced α-amylase only, and not β-amylase as there was very little maltose found in the developed chromatogram. However, some maltase, or glucoamylase capable of acting on maltose, was present (Fig. 4.1), and this may have concealed the presence of β-amylase.

When E. fibuliger was grown in symbiosis with C. utilis (Fig. 4.2), the carbohydrate changes were different from those of the symbiotic process, with the addition of either A. niger or A. awamori (Fig. 4.4 and 4.5). As shown in Fig. 4.2, glucose appeared only during the first two hours, then only trace quantities of glucose remained throughout the whole period of fermentation. Since E. fibuliger has been reported to produce α-and β-amylases, it might be expected that there should be large amounts of maltose and glucose released from starch during

the culture period. The chromatograms show, however, that there was very small amount of glucose present, thus indicating that C. utilis immediately used these sugars for its growth and multiplication. This is also apparent from the reducing sugar formation and depletion shown in Fig. 2.3. Evidence showed that the amount of reducing sugar present was closely related to the glucose spot on the chromatogram at the 8th hour of yeast propagation.

When the fermented mash of A. awamori was used as the enzyme solution to convert starch to sugars, the paper chromatogram showed the activities of both α-amylase and glucoamylase. From Fig. 4.1., it could definitely be concluded that A. awamori excretes α-amylase and glucoamylase, and that the activity of the latter was much stronger. When A. awamori was grown in symbiosis with C. utilis, a large amount of glucose and some maltose were present during the first 4 hours (Figure 4.3). Oligosaccharide formation was also identified on the chromatogram of mash from the growth of A. awamori alone. This could lead one to conclude that A. awamori excretes α-amylase and glucoamylase whether it is grown alone or in symbiosis with C. utilis. Tsujisaka et al (57) have reported that glucoamylase isolated from A. awamori, does not hydrolyze maltose. From the present study, however, the glucoamylase excreted from A. awamori or a separate enzyme (maltase) hydrolyzed maltose to a certain degree (Fig. 4.1).

The fermented mash obtained from a symbiotic propagation

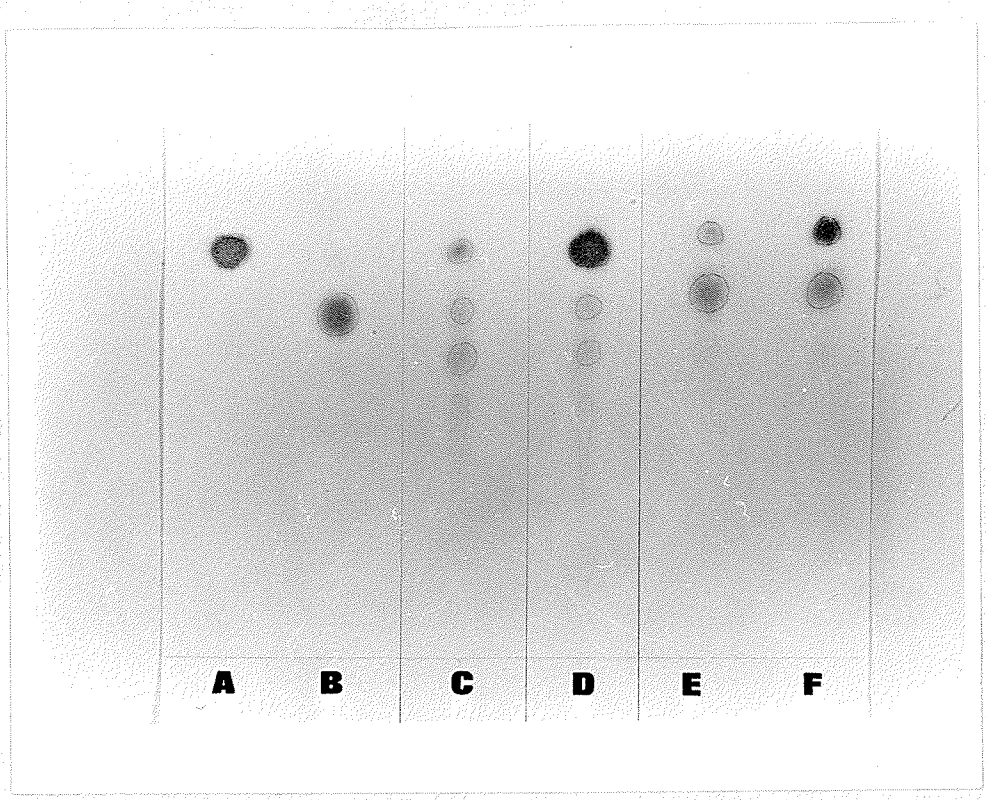


Figure 4.1. Paper chromatogram of carbohydrate changes caused by the enzymes obtained from the growth of E. fibuliger and A. awamori Individually

A. Glucose

B. Maltose (purified sample)

C. Enzymic reaction of E. fibuliger NRRL-Y 25 on a starch substrate

D. Enzymic reaction of A. awamori NRRL-3112 on a starch substrate

E. Enzymic reaction of E. fibuliger NRRL-Y 25 on a maltose substrate

F. Enzymic reaction of A. awamori NRRL-3112 on a maltose substrate

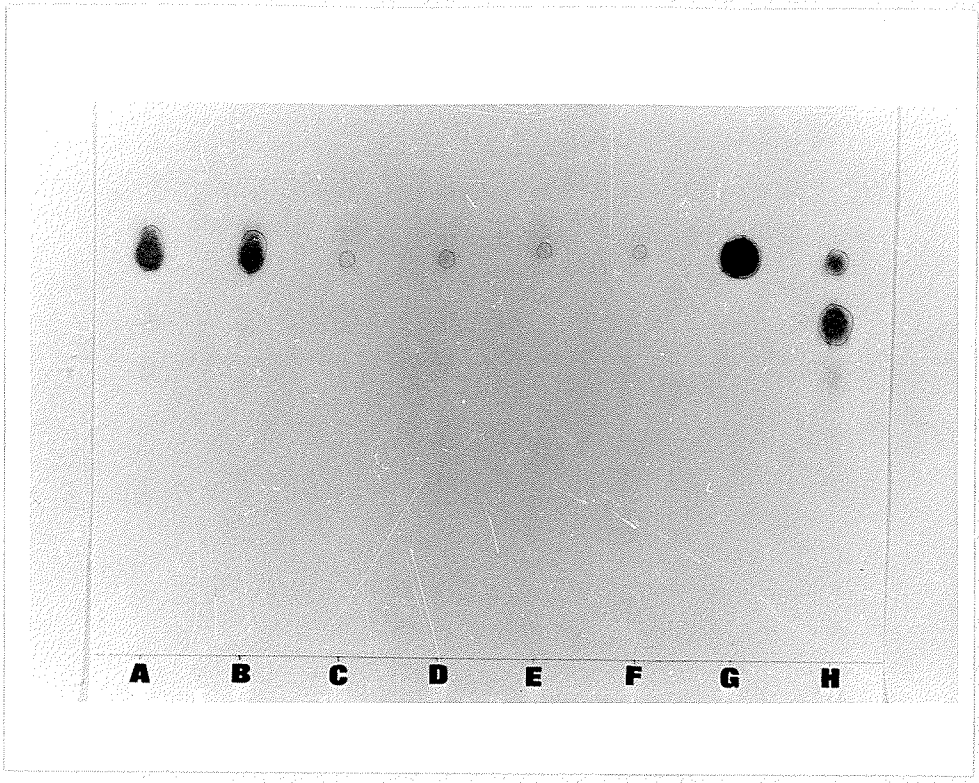


Figure 4.2. Paper chromatogram of carbohydrates in the mash during the symbiotic growth of E. fibuliger and C. utilis

A. Reaction sample taken at zero hour
B. Reaction sample taken at 2nd hour
C. Reaction sample taken at 4th hour
D. Reaction sample taken at 6th hour
E. Reaction sample taken at 8th hour
F. Reaction sample taken at 10th hour
G. Glucose
H. Maltose(Fisher reagent)

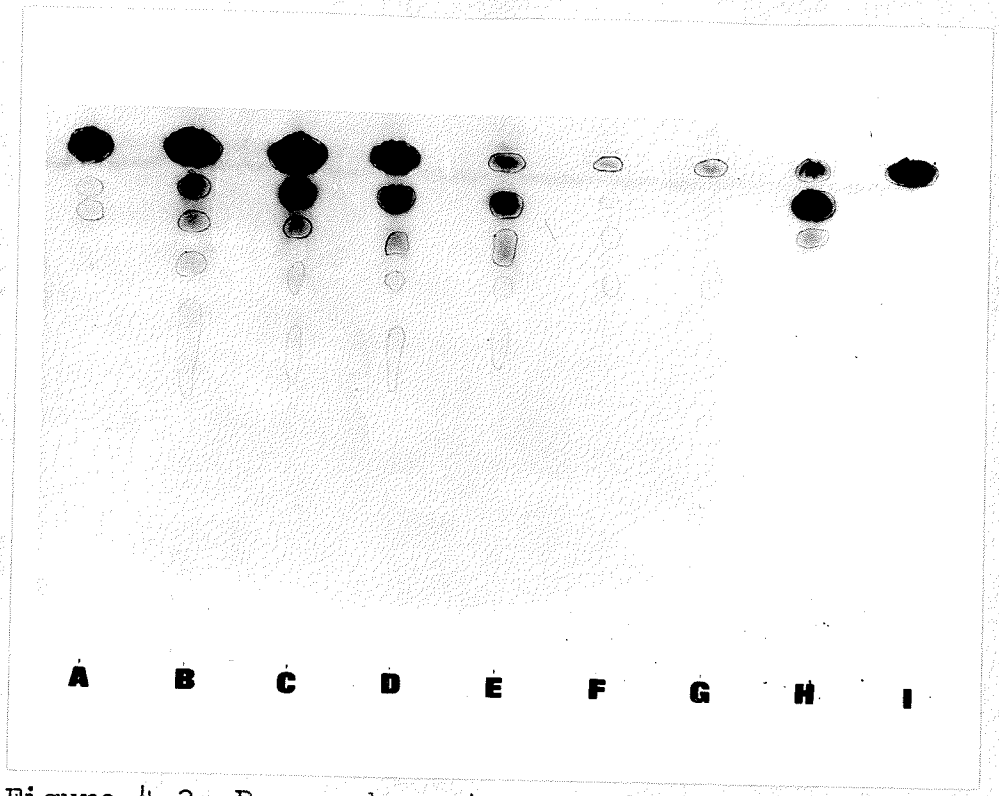


Figure 4.3. Paper chromatogram of carbohydrates in the mash during the symbiotic growth of A. awamori and C. utilis

A. Reaction sample taken at zero hour
B. Reaction sample taken at 2nd hour
C. Reaction sample taken at 4th hour
D. Reaction sample taken at 6th hour
E. Reaction sample taken at 7th hour
F. Reaction sample taken at 8th hour
G. Reaction sample taken at 10th hour
H. Maltose (Fisher reagent)
I. Glucose

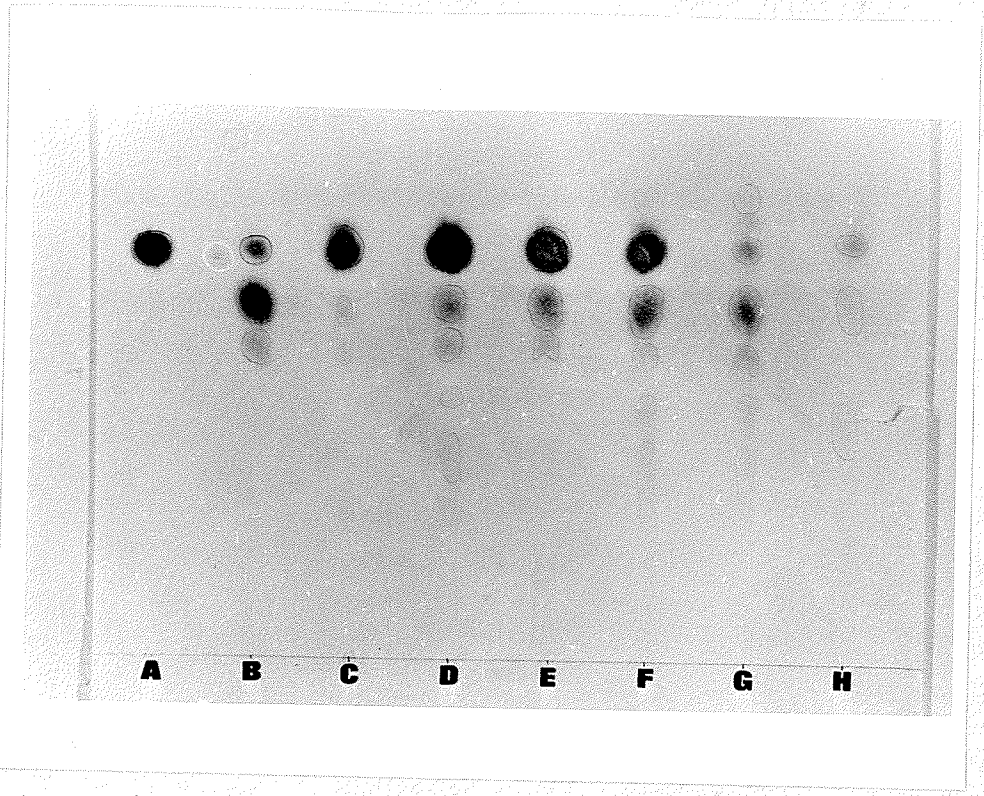


Figure 4.4. Paper chromatogram of carbohydrates in the mash during the symbiotic growth of E. fibuliger, A. awamori and C. utilis

A. Glucose
B. Maltose (Fisher reagent)
C. Reaction sample taken at zero hour
D. Reaction sample taken at 2nd hour
E. Reaction sample taken at 4th hour
F. Reaction sample taken at 6th hour
G. Reaction sample taken at 7th hour
H. Reaction sample taken at 9th hour

of C. utilis, A. niger and E. fibuliger was also studied (Fig.4.5). The pattern of the paper chromatogram is rather close to the chromatogram shown in Fig. 4.6 which was obtained from the fermented mash of the symbiotic growth of C. utilis and A. niger. According to Fig. 4.5, maltose was still present at 5th and 7th hr. fermentation mash, while it was almost disappeared at 2nd hour and later fermentation mash in Fig. 4.6. The reason for this difference is unknown.

The major difference between the A. awamori and A. niger was that more maltose was released by the enzymatic activity of A. awamori (Figs. 4.3 and 4.6, or 4.4 and 4.5). According to the chromatograms on Figures 4.5 and 4.6., it was evident that more glucose was released by the enzymatic activity of A. niger. It might be concluded that the predominance of glucoamylase or $\alpha$-amylase characterizes to a certain degree the difference between these two fungi.

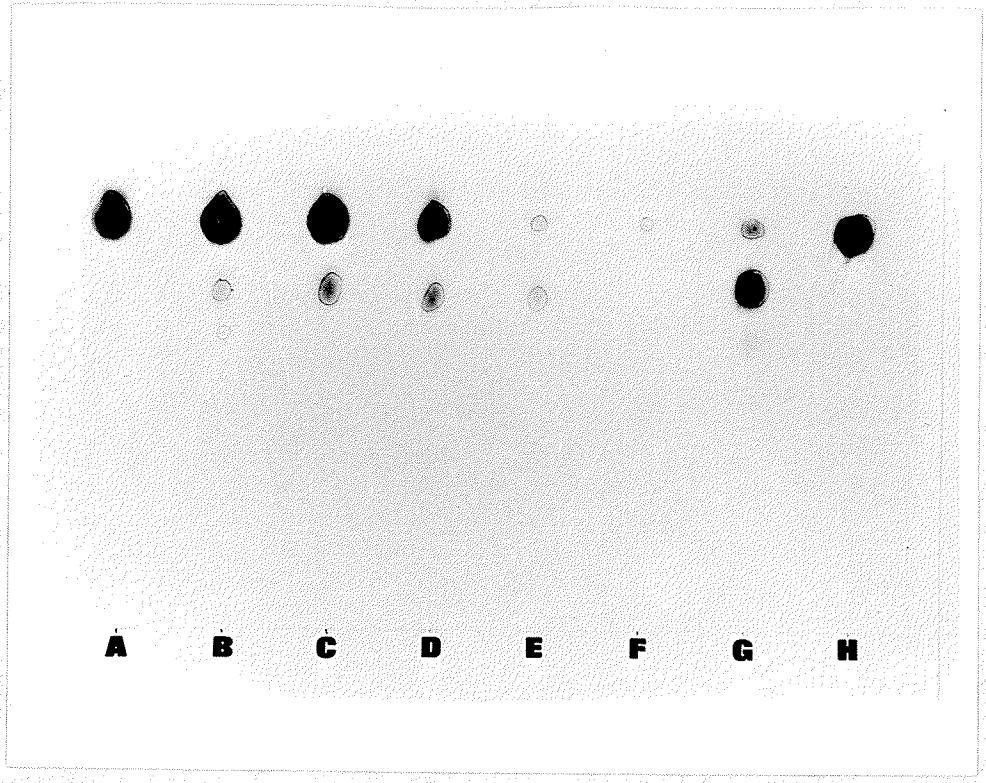


Figure 4.5. Paper chromatogram of carbohydrates in the mash during the symbiotic growth of E. fibuliger, A. niger and C. utilis

A. Reaction sample taken at zero hour
B. Reaction sample taken at 3rd hour
C. Reaction sample taken at 5th hour
D..Reaction sample taken at 7th hour
E. Reaction sample taken at 9th hour
F. Reaction sample taken at 11th hour
G. Maltose(Fisher reagent)
H. Glucose

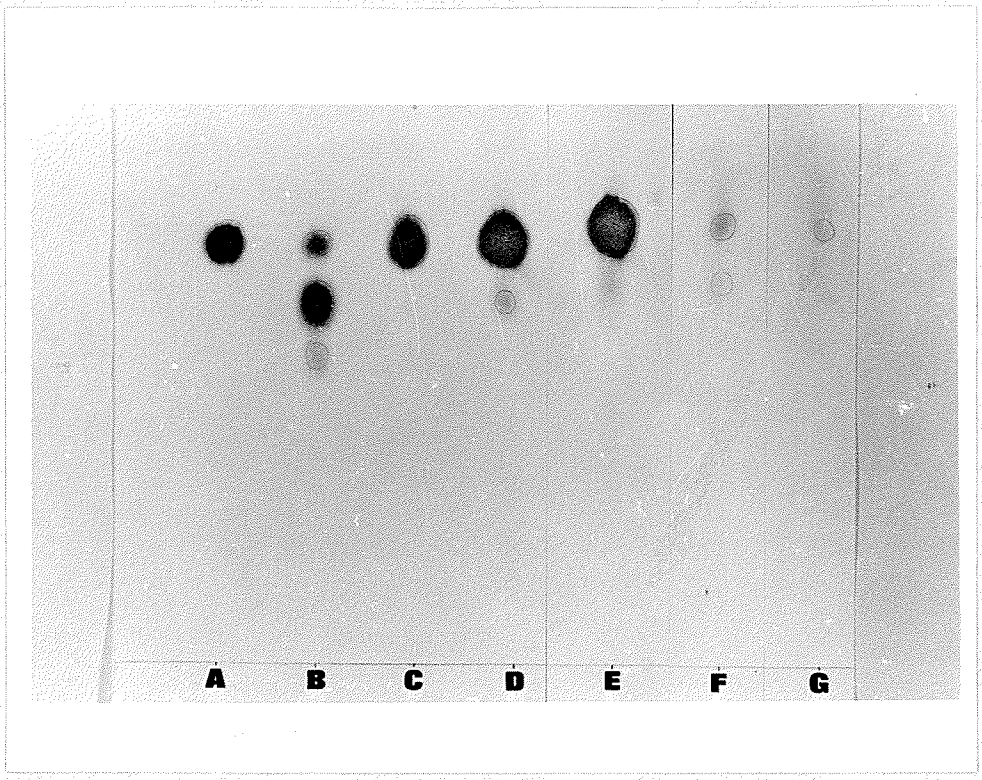


Figure 4.6. Paper chromatogram of carbohydrates in the mash during the symbiotic growth of A. niger and C. utilis

A. Glucose

B. Maltose (Fisher reagent)

C. Reaction sample taken at zero hour

D. Reaction sample taken at 2nd hour

E. Reaction sample taken at 4th hour

F. Reaction sample taken at 7th hour

G. Reaction sample taken at 9th hour

## Section IV. Qualitative Study of Protein from a Symbiotic Fermentation of Potato Mash


### Abstract

The chemical properties, amino acid composition and solubility characteristics of the product obtained from symbiotic growth with potato starch as the carbohydrate source were studied. The analytical data revealed that the product contained approximately 40% protein, 2% fat, 7.6% fiber and 45% nitrogen-free residue. The amino acid composition showed that the protein (predominantly yeast protein) was similar to soybean protein and might be used as a feed supplement.


### Introduction

Although yeasts have been used for food since ancient times in baking, the first really extensive effort to manufacture yeast on a large scale for nutritional use was made in Germany during World War II. At that time, the Germans incorporated about 16,000 tons of torula yeast per year into human foods. However, its use as a source of protein has lagged far behind existing knowledge of its nutritive value.

Investigation of the nutritive value of yeast has been mainly concentrated on chemical studies of the constituents of yeast and on animal feeding experiments. Most work has been done on torula yeast produced from molasses, wood hydrolyzate, or sulfite liquor. Kuehner and Wickerham (79) were apparently the first investigators to study the propagation and nutritive value

of yeasts grown in a symbiotic culture (the Symba process). They reported that yeast, obtained from symbiotic growth of C. utilis and E. fibuliger with potato starch as the carbohydrate source, contained approximately 35% protein; niacin 29.0, panthothenic acid 11.0, riboflavin 1.0, biotin 0.10, pyridoxine 3.0, thiamine 10.0 and choline 1.1 mcg per g dry material; as well as arginine 22.8, leucine 20.4, phenylalanine 11.2, valine 31.6, histidine 7.6, threonine 60, isoleucine 36.4 and methionine 2.4 mcg per g of dry material. Jarl (84), using the same procedure and microorganisms, found that the Symba yeast contained 7% moisture, 40 and 50% crude protein, approximately 5% fiber, 5% ash and approximately 4 to 5% lipid. No amino acid composition or vitamin content of Symba yeast was reported by Jarl.

The nature of yeast protein was investigated by Thomas (42, 43, 44, 45) from 1913 to 1921. He obtained two proteins by aqueous extraction of yeast, the yield being greatest if the extraction was carried out at 35°C. in weak alkali medium. One part phosphoprotein to three parts true albumin, were found. The phosphoprotein, zymocasein, was insoluble in water, soluble in alkali, and contained 16.2% N and 1.8% P. It was coagulated by rennet. The albumin, cerevisin, was soluble in water and coagulated in stages between 41° and 70°C. It contained 16.4% N and 0.9% S. Since then, numerous publications have reported investigations on different enzymes from yeast products, but none seems to have dealt with the nature of yeast protein.

It is the purpose of this paper to investigate the proximate analysis, amino acid composition and solubility characteristics of protein from symbiotic process involving three microorganisms, and to assess the feasibility of using the protein as a supplement in poor-quality foods, such as the cereal grains, and in the preparation of food or feed mixtures with a high-protein content.

Materials and Methods

Products were obtained from symbiotic cultures involving the following combinations of organisms: C. utilis, E. fibuliger and A. awamori; C. utilis, E. fibuliger and A. niger; C. utilis and E. fibuliger; C. utilis and A. awamori; C. utilis and A. niger; and C. utilis grown alone on HCl-hydrolyzate of potato mash. The preparation of raw material and the procedures for symbiotic culture were as described in Section I. The product was harvested by centrifuging the fermented mash at 3000 r.p.m. for 10 minutes. After decanting the supernatant, the precipitate was washed with distilled water and then centrifuged again at 3000 r.p.m. for 10 minutes. After repeating the washing procedure three times, the precipitated solid was dried by a Virtis freeze drier, Model 10-145MR-DA, and preserved for analysis.

The methods used to determine the chemical composition of these products, mainly crude protein, crude fat, crude fiber, minerals and moisture, were chosen from the "Methods of Analysis" by A.O.A.C. (85).

The determination of amino acid composition of the products was conducted by weighing 100 mg into each of several test tubes and adding 5 ml of 6N hydrochloric acid to each. The tubes were evacuated and sealed under vacuum. Hydrolysis was carried out at 120°C. for 16 hours. The hydrolysate was dried over sodium hydroxide pellets in a dessicator. The residue was dissolved in 5 ml of 0.1 N HCl and then filtered. A Beckman Model 121 amino acid analyzer was used for the amino acid analysis.

The extraction procedure employed for protein was similar to the classical protein fractionation procedure of Osborne (89), except that an autolysis procedure was carried out before using Osborne's procedure. The extraction procedure unless specially noted was carried out in a cold room at 4°C. Samples (10 g dry basis) were each ground with 40 ml of 0.01 M $NaHCO_3$ and 20 g of glass beads in a Mini blender for 1 hour at 15°C., the suspension was centrifuged for 5 minutes at 400 r.p.m. for the removal of the glass beads. The supernatant suspension was autolyzed for 8 hours at 40°C. and then stirred for 24 hours in a cold room. This preparation was autolyzed again for 17 hours at 40°C. and then centrifuged for 30 minutes at 17,000 x g and the supernatant was decanted. The precipitated solids were extracted with 40 ml of 0.5 M NaCl solution by stirring with a magnetic stirrer in a centrifuge bottle for 2 hours. This suspension was again centrifuged for 30 minutes at 17,000 x g, and the supernatant was decanted. This was followed by a second similar extraction with

40 ml of 0.5 M NaCl for 1 hour. The residue was extracted with 40 ml distilled water for 30 minutes to remove residual salt. The four supernatants were combined. This extract was dialyzed against cold distilled water for 48 hours and centrifuged to separate the precipitated salt-soluble proteins; water-soluble proteins remained in the supernatant. The residual remaining after extraction with salt solution was then extracted similarly with two portions of 70% ethanol. The ethanol was removed from the combined ethanol supernatants in a rotary evaporator. The resulting residue from alcohol extraction was further extracted with two portions of 0.05% acetic acid solution. The acid extract was dialyzed against cold distilled water for 48 hours. The four soluble fractions and the final residue were freeze-dried and stored in a deep freeze.

The semi-micro Kjeldahl method was used to determine nitrogen contents of the protein extracts and the residues. Protein contents were calculated by use of the conversion factor 6.25.

Results and discussion

The chemical composition of the products from symbiotic cultures on potato mash is presented in Table 5.1. Among the mixed products from symbiotic cultures, that from a culture of C. utilis, E. fibuliger and A. awamori, had the highest, and that of C. utilis and E. fibuliger the lowest protein content. The protein content obtained from the symbiotic growth of C. utilis and A. awamori was higher than that obtained from the symbiotic growth of C. utilis and A. niger. The symbiotic growth of C. utilis and E. fibuliger

Table 5.1. Chemical Composition of Products Obtained from Symbiotic Cultures on Potato Mash*

| Yeast product from | Moisture % | Crude protein % | Crude fat % | Crude fiber % | N-free residue % | Ash % |
|---|---|---|---|---|---|---|
| *C. utilis* on HCl-hydrolyzate | 3.4 | 44.7 | 2.1 | 2.6 | 45.4 | 5.2 |
| *C. utilis*, *A. awamori* & *E. fibuliger* | 3.5 | 40.5 | 2.1 | 7.6 | 44.8 | 5.0 |
| *C. utilis*, *A. niger* & *E. fibuliger* | 3.4 | 36.6 | 2.0 | 7.8 | 48.3 | 5.3 |
| *C. utilis* & *E. fibuliger* | 3.5 | 32.8 | 2.1 | 7.9 | 52.2 | 5.0 |
| *C. utilis* & *A. awamori* | 3.4 | 39.9 | 2.1 | 7.7 | 45.2 | 5.1 |
| *C. utilis* & *A. niger* | 3.4 | 37.3 | 2.1 | 7.7 | 47.7 | 5.2 |

* Crude protein, crude fat, crude fiber, N-free residue and ash are reported as percent of dry matter.

produced a product with a low protein content due to the incomplete conversion of potato starch. C. utilis grown alone in HCl-hydrolysed potato mash had the highest protein content of any of these products, but would not have industrial feasibility because of the cost of acid hydrolysis. From the comparison of the protein contents of the final products, it could be concluded that the symbiotic culture of C. utilis, A. awamori and E. fibuliger offers an efficient process for producing yeast protein without the acid-hydrolysis of starch, and yields a product of high protein content from potatoes or potato waste.

Jarl (84) reported that his Symba yeast contained approximately 40-50% crude protein, but the fermentation time of his process was over 20 hours. The rate of protein synthesis in Jarl's procedure is approximately half that of the present study. Since unpeeled whole potatoes were used throughout the current study, it is understandable that the crude fiber content of the final mixed product would be rather high. The crude fiber content of Symba yeast described by Jarl (84) was about 5% lower than found in this study, because he screened out the larger particles from the original raw material, thus reducing the fiber content of the centrifugally harvested product. The yeast, C. utilis, is not a fat-producing strain. There was no significant difference in crude fat content of the final product obtained from the various combinations of organisms. If a high-fat yeast propagation is desired, a symbiotic growth of Rototurola, E. fibuliger and A. awamori is suggested.

The ash content of the final mixed product was approximately 5%, and this agreed with the results obtained by Jarl (84). Inskeep et al (18) reported that the ash content of yeast grown on spent sulfite liquor was about 9%, higher than in this study. From a nutritional point of view, the ash content of a product is not a major consideration. In addition, different raw materials used to grow C. utilis will give different ash contents of the final products. If a high ash content is preferred, the addition of beet molasses to the fermentation mash will increase the ash content in the final product.

The amino acid contents of products from the symbiotic growth of different mixed cultures on potato mash is presented in Table 5.2. Block and Bolling (38) presented data on the amino acid composition of several strains of yeast, which indicate that the amount of several amino acids will vary with the strain. Fink and Just (39) have reported considerable variation in amino acid content of the same type of yeast grown under slightly different conditions. Peppler (41) reported on the amino acid composition of yeast grown on different spent sulfite liquors; the variation found was relatively small for all amino acids except cystine. In the present study, only glutamic acid and arginine were found to be higher in the yeast grown in HCl-hydrolyzate of potato mash than in the mixed (symbiotic) products. The content of the other amino acids did not vary to any great extent, regardless of the type of raw material used or the type of culture employed.

Table 5.2 Amino acid content of products from symbiotic cultures grown on potato mash.

mg per g of N

| | Product obtained from | | | | |
|---|---|---|---|---|---|
| | 1* | 2* | 3* | 4* | 5* |
| Lysine | 359 | 338 | 412 | 361 | 332 |
| Histidine | 104 | 102 | 108 | 103 | 103 |
| Arginine | 364 | 263 | 287 | 298 | 274 |
| Aspartic acid | 560 | 566 | 571 | 575 | 530 |
| Threonine | 308 | 310 | 298 | 306 | 272 |
| Serine | 249 | 271 | 261 | 265 | 244 |
| Glutamic acid | 1001 | 714 | 807 | 988 | 857 |
| Proline | 202 | 202 | 203 | 205 | 185 |
| Glycine | 246 | 257 | 259 | 254 | 238 |
| Alanine | 348 | 373 | 388 | 359 | 329 |
| Valine | 320 | 330 | 331 | 352 | 282 |
| Methionine | 66 | 77 | 70 | 66 | 63 |
| Isolucine | 272 | 272 | 284 | 270 | 243 |
| Leucine | 418 | 442 | 459 | 429 | 398 |
| Tyrosine | 163 | 199 | 168 | 176 | 165 |
| Phenylalanine | 234 | 262 | 260 | 252 | 233 |
| N % recovery | 79.4 | 78.4 | 79.4 | 80.8 | 78.9 |

*

1. C. utilis grown on HCl-hydrolyzate
2. Symbiotic growth of C. utilis, A. awamori and E. fibuliger
3. Symbiotic growth of C. utilis and A. awamori
4. Symbiotic growth of C. utilis and A. niger
5. Symbiotic growth of C. utilis, A. niger and E. fibuliger

Over the last 50 years, a large number of different biological procedures have been suggested for the measurement of protein quality. Results obtained by biological methods are a function of the limiting amino acid in the diet. The rate of growth of an animal under controlled conditions is related to the quality of the dietary protein, and has been used widely to evaluate proteins in foods. Therefore, it is suggested that the evaluation of protein quality of the product obtained from this study should be done by biological procedures.

The percentage of nitrogen recovery in this study was about 79% as shown in Table 5.2. This is in agreement with the results of several investigators who showed that approximately 80% of the nitrogen of yeast crude protein is protein nitrogen.

The amino acid content of a protein analysed by chemical methods has also been employed to evaluate the nutritional value of protein, although such effects as the digestibility or the processed protein or the availability of amino acids are not taken into account. The comparison of essential amino-acid content of yeast and other proteins is presented in Table 5.3. The amount of various essential amino acids in protein obtained from this study was similar to that of soybean protein although it was consistently lower than that of torula yeast obtained from other sources.

This subject of protein-nitrogen composition takes on the greatest importance if yeast is looked upon as a cheap source

Table 5.3. Essential amino acid content of yeast and other proteins, mg per g of N

| Amino acid | This study* | Torula | Brew-er's | Casein | Cotton seed | Soybean | Egg |
|---|---|---|---|---|---|---|---|
| Tryptophan | | 86 | 96 | 84 | 74 | 86 | 103 |
| Threonine | 272 | 315 | 318 | 269 | 221 | 246 | 311 |
| Isoleucine | 243 | 449 | 324 | 412 | 236 | 336 | 415 |
| Lysine | 332 | 493 | 446 | 504 | 268 | 395 | 400 |
| Leucine | 398 | 501 | 436 | 632 | 369 | 482 | 550 |
| Total sulfur amino acids | (63)# | 153 | 187 | 218 | 188 | 195 | 342 |
| Phenylalanine | 233 | 319 | 257 | 339 | 327 | 309 | 361 |
| Valine | 282 | 392 | 368 | 465 | 308 | 328 | 464 |
| Arginine | 274 | 451 | 304 | 256 | 702 | 452 | 410 |
| Histidine | 103 | 169 | 169 | 190 | 166 | 149 | 150 |

All sources except that of "this study" are from Hochberg et al (1945), J. Nutrition 30: 201.

* Product obtained from the symbiotic growth of C. utilis, E. fibuliger and A. niger

\# Only methionine was determined

of protein to supplement poor-quality protein foods or to serve as components of protein-rich foods. Csonba (35) reported values for tryptophan, histidine, lysine and arginine in yeast and indicated that they were present in adequate amounts to supply the requirements of the rat, as indicated by Rose (36). Skinner and Muller (37) showed that the protein of yeast and of other molds was relatively poor in the sulfur-containing amino acids, as had been reported before. Lindan and Work (27), and Spanyer and Thomas (40) studied the sulfur-containing amino acids in baker's and brewer's yeasts, and found both yeasts to be low in methionine and cystine. From the present study, methionine was found to be low in the final product of the Symba process. Additional amount of methionine should be added when the product is employed as a feed supplement.

The solubility characteristics of protein obtained from the symbiotic growth of C. utilis, E. fibuliger and A. awamori, and of C. utilis grown alone on HCl-hydrolyzate of potato mash are presented in Table 5.4. The solubility characteristics of proteins has been investigated mostly in grains or cereals; little work has been done on yeast protein so far. Thomas (42, 43, 44, 45) conducted experiments on the nature of yeast protein, and reported that two proteins were obtained on aqueous extraction of yeast. One part phosphoprotein to three parts true albumin were found. In the current study, no attempt was made to fractionate the soluble protein, but it could be assumed that the

aqueous fraction of this study also contained phosphoprotein and albumin. The fractional composition for yeast protein obtained from a pure microbial process of C. utilis grown alone on HCl-hydrolyzate of potato mash was rather similar to that obtained from the symbiotically grown product, but the alcoholic fraction of C. utilis when grown in the HCl-hydrolyzate of potato was slightly larger than that of the symbiotic product.

Table 5.4 Solubility characteristics of proteins

| | Protein obtained from | |
|---|---|---|
| | I | II |
| Water-soluble fraction | | |
| Weight, mg | 1391.7 | 1561.0 |
| Protein content % | 31.1 | 31.2 |
| Fraction of total protein, % | 10.7 | 10.9 |
| Salt-soluble fraction | | |
| Weight, mg | 115.9 | 211.8 |
| Protein content % | 47.9 | 44.5 |
| Fraction of total protein, % | 1.4 | 2.1 |
| Alcohol-soluble fraction | | |
| Weight, mg | 699.9 | 823.4 |
| Protein content % | 45.2 | 47.3 |
| Fraction of total protein, % | 7.8 | 8.7 |
| Acid-soluble fraction | | |
| Weight, mg | 209.6 | 245.6 |
| Protein content % | 42.9 | 43.6 |
| Fraction of total protein, % | 2.2 | 2.4 |
| Residue | | |
| Weight, mg | 6655 | 6521.1 |
| Protein content % | 36.8 | 38.8 |
| Fraction of total protein, % | 60.4 | 56.6 |
| Nitrogen recovery, % | 82.5 | 80.7 |

I. Product obtained from the symbiotic growth of C. utilis, E. fibuliger and A. awamori

II. C. utilis grown on HC1-hydrolyzate of potato mash

# SUMMARY

A process has been developed in which a mixed culture consisting of amylase-producing microorganisms, Endomycopsis fibuliger and Aspergillus awamori, and non-amylase-producing yeast such as Candida Utilis were cultured on a cooked, but not converted, potato mash. The entire mass of centrifugable solids from the culture was harvested as product. This product, containing yeast, mould and residual material form the substrate used, contains nutritionally valuable material such as protein and various vitamins.

In general, yields of approximately 75 pounds of solids were obtained from 100 pounds of potato starch, or 667 pounds of fresh potato equivalent. The protein in these solids varied from 32.8-45.3%. An effort to determine the optimum operating conditions for the process was made by changing a single organism in any one series of experiments.

The optimum concentration of potato mash was 4 to 5%. Urea and ammonium sulfate, incorporated in the mash as nitrogen sources, were added to the mash simultaneously. The mixed nitrogen source had an advantage in that it was capable of maintaining a constant pH, thus reducing the need for a neutralizing agent such as sodium hydroxide or sulfuric acid. Although the fermentation process may operate over a broad range of pH values, the optimum was found to be at pH 4.5.

The symbiotic growth of E. fibuliger, A. awamori and

C. utilis gave better yields of protein and total yields of product than the other combinations consisting of A. niger, E. fibuliger and C. utilis; A. awamori and C. utilis; A. niger and C. utilis or E. fibuliger and C. utilis. The symbiotic growth of E. fibuliger and C. utilis, according to this study, gave the poorest results, on the bases of protein yield and total product yield. All cultures were grown in a modified Waldhof fermentor in the presence of a source of assimilable nitrogen.

Evidence was obtained that the symbiotic growth of microorganisms such as E. fibuliger, C. utilis and A. awamori could be adapted to a continuous process. Two experiments on continuous and symbiotic growth, with the same organisms under similar conditions, were conducted in this study. A potato mash pre-treated with E. fibuliger and A. awamori when used as a mash for continuous fermentation gave a significantly better yield of protein, and greater carbohydrate utilization, than did a non-treated potato mash. The product, contained 42.1% crude protein, the carbohydrate utilization was 90.4%, and the yield was 73% based on the average initial concentration of starch. The exchange rate during continuous culture was approximately 17% (V/V) per hour, thus the effective fermentor volume was enlarged four-fold over that of the same fermentor operated on the batch basis (one batch each 24 hours). Approximately 67 hours continuous and symbiotic growth was achieved in this study.

The change in carbohydrates in the symbiotic process

with potato starch substrate was investigated. Ascending paper chromatography was conducted to identify the carbohydrates present every 2 hours during batch growth. Major attention was paid to the conversion of starch to glucose, maltose and oligosaccharide. Maltose and glucose were found to be the major end products of the amylase action of E. fibuliger, and these in turn were consumed by C. utilis during the fermentation. Glucose and dextrins, on the other hand, were formed during the symbiotic propagation of C. utilis and E. fibuliger. No strong evidence could be found to prove that E. fibuliger excreted both $\alpha$-amylase and $\beta$-amylase during its growth. Glucose was found to be the main carbohydrate end product during the symbiotic growth of A. awamori or A. niger and C. utilis. Maltose was formed as an intermediate during the conversion of starch to dextrins, and then degraded to glucose. This agreed with the general conclusion that A. awamori or A. niger excret both $\alpha$-amylase and glucoamylase. No attempt was made to identify the $\alpha$-amylase activity in the culture of E. fibuliger, A. awamori or A. niger. Evidence for dextrins and oligosaccharides obtained from paper chromatography, however, showed that $\alpha$-amylase was excreted by either E. fibuliger, A. awamori or A. niger during the symbiotic growth with C. utilis.

The nutritional value of the mixed product from this study was evaluated by proximate analysis and amino acids composition; and the solubility characteristics of yeast protein were also determined. No significant variation was found in the proximate

analyses, except that variation in the crude protein content was found among the products obtained from different combinations of organisms. The amount of crude protein, which was given the most attention, was found to be highest in the product of the symbiotic growth of E. fibuliger, A. awamori and C. utilis, with the exception that the yeast product obtained from the growth of C. utilis on HCl-hydrolyzate of potato starch gave higher results.

The amino acid composition of the product was determined by a Beckman amino acid autoanalyzer. The variation in amino acid composition of different products obtained from different combinations of microorganisms using the same substrate was not significant. On the basis of the amount and composition of amino acids, it could be concluded that the quality of the protein of this product is very similar to the quality of soybean protein.

From the results of the solubility characteristics of yeast protein, the protein was found to be made up of the following fractions: water-soluble fraction, 10.7%; salt-soluble fraction 1.4%; alcohol-soluble fraction, 7.8%; acid-soluble fraction 2.2%; and residual fraction 60.4%. Since most of the yeast protein remained in the residual fraction, it could be assumed that the yeast product is not suitable for protein extraction but may be used as an animal feed supplement.

## CONTRIBUTIONS TO KNOWLEDGE

1. A high protein product was produced from potato starch with a mixture culture of E. fibuliger, A. awamori and C. utilis. The fermentation mash consisted of unpeeled and ground potatoes, urea, ammonium sulfate and potassium phosphate. Batch fermentation was conducted at 33 to 35°C. and pH 4.5 for 8 to 10 hours under vigorous aeration and agitation. The yield of final product was 75% based on the initial concentration of starch in the fermentation mash; and the crude protein content of the product was approximately 40% on a dry weight basis.
2. Under identical fermentation conditions, a high protein product was produced directly by a symbiotic and continuous process. The microorganisms employed and the fermentation mash used were identical to the above description, except that the potato mash had to be pretreated by a mixed culture of E. fibuliger and A. awamori (20:80) at 55°C. for 2 hours. The average yield of finished product was 73% based on the average concentration of starch in the continuous fermentation mash; and the average crude protein content was approximately 42% on a dry weight basis. An exchange rate of 15 to 17% (V/V) of available fermenting volume was obtained during 68 hours of continuous fermentation.
3. The finished product obtained from such a symbiotic and batch process, as described in section 1, contained the following composition:

A. Proximate analysis (dry base):

| | | | |
|---|---|---|---|
| Crude protein | 40.5% | Nitrogen-free residue | 44.8% |
| Crude fiber | 7.6% | Minerals | 5.0% |
| Crude fat | 2.1% | | |

B. Amino acid composition, mg/g N

| | | | |
|---|---|---|---|
| Lysine | 338 | Histidine | 102 |
| Arginine | 263 | Aspartic acid | 566 |
| Threonine | 310 | Serine | 271 |
| Glutamic acid | 714 | Proline | 202 |
| Glycine | 257 | Alanine | 373 |
| Valine | 330 | Methionine | 77 |
| Isoleucine | 272 | Leucine | 442 |
| Tyrosine | 199 | Phenylalanine | 262 |

C. Protein solubility characteristics

| | |
|---|---|
| Water-soluble fraction | 10.7% |
| Salt-soluble fraction | 1.4% |
| Alcohol-soluble fraction | 7.8% |
| Acid-soluble fraction | 2.2% |
| Residue | 60.4% |

4. According to the results of paper chromatography of fermented mash, data of this investigation suggested that E. fibuliger excreted $\alpha$-amylase, but not $\beta$-amylase, during its growth. Data also suggested that A. awamori and A. niger both excreted glucoamylase during their growth in symbiosis with C. utilis or alone.

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# APPENDIX

Table 2.1 The growth of yeast on potato HCl-hydrolyzate.

| | Trial | Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Yeast population ($\times 10^{-6}$/ml) | 1 | 110 | 200 | 300 | 550 | 800 | 800 |
| | 2 | 90 | 200 | 325 | 475 | 750 | 1050 |
| | average | 100 | 200 | 313 | 512 | 775 | 925 |
| Sugar content mg/100 ml | 1 | 3780 | 3680 | 2461 | 1288 | 752 | 231 |
| | 2 | 4488 | 3363 | 2212 | - | 799 | 345 |
| | average | 4134 | 3521 | 2337 | 1288 | 776 | 288 |

Table 2.2 The growth of yeast and the change in carbohydrate content of the medium during the symbiotic growth of E. fibuliger and C. utilis

| | Trial | Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Yeast population ($\times 10^{-6}$/ml) | 1 | 68 | 100 | 300 | 650 | 700 | |
| | 2 | 100 | 100 | 220 | 460 | 650 | |
| | average | 84 | 100 | 260 | 555 | 675 | |
| Starch & dextrin * mg/100 ml | 1 | 4895 | 3079 | 2354 | 1840 | 2035 | |
| | 2 | 3505 | 3875 | 2213 | 1516 | 1402 | |
| | average | 4200 | 2977 | 2284 | 1678 | 1719 | |
| Reducing sugar mg/100 ml | 1 | 257 | 1326 | 1024 | 456 | 179 | |
| | 2 | 211 | 1216 | 856 | 354 | 158 | |
| | average | 234 | 1271 | 940 | 405 | 169 | |
| Iodine-test | | blue | blue | blue-violet | blue-violet | violet | |

* glucose equivalent

Table 2.3. The growth of yeast and the change in carbohydrate content of the medium during the symbiotic growth of E. fibuliger, A. awamori and C. utilis

| | Trial | Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Yeast population ($\times 10^{-5}$/ml) | 1 | 100 | 100 | 250 | 650 | 900 | 900 |
| | 2 | 80 | 90 | 170 | 450 | 800 | 850 |
| | 3 | 150 | 150 | 250 | 350 | 980 | 1000 |
| | 4 | 75 | 125 | 440 | 750 | 950 | 1050 |
| | 5 | 100 | 100 | 165 | 1000 | 1250 | 1600 |
| | average | 101 | 111 | 255 | 640 | 976 | 1080 |
| Starch & dextrin * mg/100 ml | 1 | 3762 | 3340 | 2756 | 618 | 246 | |
| | 2 | 2988 | 1332 | 282 | 252 | 201 | |
| | 3 | 4383 | 3202 | 2454 | 301 | 11 | |
| | 4 | 4272 | | | | 285 | |
| | 5 | 3067 | 2453 | 1312 | 548 | 255 | |
| | average | 3874 | 2582 | 1701 | 429 | 200 | |
| Reducing sugar mg/100 ml | 1 | 156 | 1249 | 1310 | 114 | 73 | |
| | 2 | 187 | 1568 | 2052 | 188 | 84 | |
| | 3 | 153 | 1378 | 1334 | 258 | 165 | |
| | 4 | 165 | | | | 134 | |
| | 5 | 151 | 2169 | 1037 | 104 | 82 | |
| | average | 162 | 1591 | 1433 | 166 | 108 | |
| Iodine-test | | blue | violet | violet | brown | brown | light brown |

* Glucose equivalent

Table 2.4. The growth of yeast and the change in carbohydrate content of the medium during the symbiotic growth of *E. fibuliger*, *A. niger* and *C. utilis*

| | Trial | Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Yeast population ($\times 10^{-6}$/ml) | 1 | 50 | 120 | 300 | 850 | 1000 | 1000 |
| | 2 | 80 | 80 | 125 | 250 | 500 | 800 |
| | 3 | 40 | 40 | 80 | 320 | 600 | 1000 |
| | average | 57 | 80 | 168 | 473 | 700 | 933 |
| Starch & dextrin * mg/100 ml | 1 | 3876 | 1927 | 1322 | 747 | 323 | 311 |
| | 2 | 4264 | 912 | 786 | 286 | 315 | 345 |
| | 3 | 4482 | | | | | 668 |
| | average | 4207 | 1420 | 1054 | 517 | 319 | 441 |
| Reducing sugar mg/100 ml | 1 | 152 | 1751 | 2356 | 849 | 247 | 138 |
| | 2 | 184 | 1609 | 2103 | 971 | 187 | 101 |
| | 3 | 195 | | | | | 185 |
| | average | 177 | 1680 | 2230 | 910 | 217 | 141 |
| Iodine-test | | blue | violet | violet | brown | brown | brown |

* glucose equivalent

Table 2.5. The growth of yeast and the change in carbohydrate content of the medium during the symbiotic growth of A. niger and C. utilis

| | Trial | Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Yeast population ($\times 10^{-6}$/ml) | 1 | 100 | 150 | 500 | 800 | 1050 | |
| | 2 | 80 | 80 | 160 | 520 | 750 | 875 |
| | 3 | 75 | 75 | 110 | 250 | 500 | 750 |
| | average | 85 | 102 | 257 | 523 | 767 | 813 |
| Starch & dextrin * mg/100 ml | 1 | 4037 | 2488 | 1096 | 825 | 668 | 368 |
| | 2 | 4293 | 2037 | 1093 | 1173 | 239 | 214 |
| | 3 | 2690 | 1268 | 620 | 414 | 208 | 200 |
| | average | 3673 | 1931 | 936 | 804 | 372 | 261 |
| Reducing sugar mg/100 ml | 1 | 235 | 1246 | 326 | 249 | 148 | 154 |
| | 2 | 221 | 1213 | 2169 | 1101 | 157 | 102 |
| | 3 | 87 | 1268 | 1626 | 1040 | 184 | 96 |
| | average | 181 | 1242 | 1374 | 797 | 163 | 117 |
| Iodine-test | | blue | violet | violet | brown | brown | brown |

* glucose equivalent

Table 2.6. The growth of yeast and change in carbohydrate content of the medium during the symbiotic growth of A. awamori and C. utilis

| | Trial | Time (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 2 | 4 | 6 | 8 | 10 |
| Yeast population ($\times 10^{-6}$/ml) | 1 | 80 | 100 | 260 | 500 | 920 | 920 |
| | 2 | 75 | 75 | 170 | 425 | 800 | 1000 |
| | 3 | 80 | 85 | 200 | 750 | 950 | 950 |
| | average | 78 | 85 | 210 | 558 | 890 | 957 |
| Starch & dextrin * mg/100 ml | 1 | 4350 | 2639 | 1720 | 1360 | 227 | 193 |
| | 2 | 3898 | 2610 | 2227 | 435 | 726 | 107 |
| | 3 | 3452 | 1224 | 1196 | | 268 | 231 |
| | average | 3900 | 2158 | 1714 | 898 | 407 | 177 |
| Reducing sugar mg/100 ml | 1 | 309 | 2411 | 1304 | 493 | 279 | 279 |
| | 2 | 248 | 980 | 947 | 1507 | 52 | 52 |
| | 3 | 208 | 1971 | 2081 | | 146 | 104 |
| | average | 255 | 1787 | 1444 | 1000 | 159 | 145 |
| Iodine-test | | blue violet | | brown | brown | brown | brown |

* glucose equivalent

Table 3.1. Data from a symbiotic and continuous process using a non-amylase-treated starch mash as carbohydrate source.

| Time Hr. | Yeast population x $10^{-6}$/ml | Starch & dextrin* mg/100 ml | Reducing sugar mg/100 ml | Exchange rate 1/h per 8 l fermented mash | Note |
|---|---|---|---|---|---|
| 0 | 40 | 4998 | 1249 | | |
| 2 | 40 | 1118 | 416 | | |
| 4 | 100 | | | | |
| 6 | 250 | | | | |
| 8 | 700 | | | | |
| 10 | 1000 | 1118 | 238 | 1.17 | Started continuous feeding |
| 12 | 950 | | | | |
| 14 | 1000 | 1145 | 236 | | |
| 16 | 900 | | | | |
| 18 | 1000 | 1057 | 211 | | |
| 22 | 1050 | 990 | 237 | | |
| 24 | 1125 | | | | |
| 26 | 1125 | | | | |
| 27 | 1250 | 1040 | 267 | 2.0 | |
| 29 | 1250 | | | | |
| 31 | 1305 | 1112 | 240 | | |
| 33 | 1070 | | | 1.2 | |
| 37 | 625 | 1206 | 268 | 2.0 | Stopped feed |
| 38 | 1000 | | | | |
| 40 | 1000 | 1249 | 244 | Re-started feed | |
| 43 | 750 | Contamination | | | |
| 47 | 625 | 1298 | 174 | Contamination | |
| 48 | 875 | | | Contamination | |
| 49 | 500 | Stopped feed | | | |
| 51 | 1000 | | | | |
| 53 | 1000 | 969 | 135 | Re-started feed | |
| 55 | 1025 | | | | |
| 57 | 1050 | 766 | 151 | | |
| 59 | 1250 | | | | |
| 61 | 1250 | 888 | 209 | 1.1 | |
| 63 | 1750 | | | | |
| 64 | 850 | 810 | 233 | | |
| 68 | 1000 | | | Finished experiment | |

* glucose equivalent

Table 3.2. Data from a symbiotic and continuous process using an amylases-treated-starch mash as carbohydrate source

| Time Hr. | Yeast population x $10^{-6}$/ml | Starch & dextrin* mg/100 ml | Reducing sugar mg/100 ml | Exchange rate 1/h per 8 l fermented mash | Note |
|---|---|---|---|---|---|
| | 720 | 2910 | 312 | | |
| 0 | 700 | 2272 | 74 | | Started feed |
| 1 | 750 | 280 | 77 | | |
| 3 | 1125 | | | | |
| 5 | 1125 | 385 | 116 | 1.2 | |
| 7 | 1150 | | | | |
| 9 | 1275 | 394 | 196 | | |
| 11 | 1150 | | | | |
| 13 | 1125 | 411 | 181 | | |
| 15 | 1225 | | | 1.2 | |
| 17 | 900 | 386 | 134 | | |
| 19 | 1125 | | | | |
| 21 | 1000 | 418 | 128 | | |
| 23 | 1100 | | | | |
| 25 | 1850 | 414 | 113 | | |
| 27 | 1700 | | | 1.5 | |
| 29 | 1250 | 359 | 117 | | |
| 31 | 1200 | | | 1.6 | |
| 33 | 1600 | 370 | 113 | 1.35 | |
| 35 | 1100 | | | 1.5 | |
| 37 | 1189 | 317 | 113 | 1.2 | |
| 39 | 625 | Stopped feed | | | |
| 41 | 1000 | 330 | 111 | | Re-started feed |
| 43 | 1000 | | | | |
| 45 | 1000 | 304 | 99 | | |
| 47 | 900 | | | | |
| 49 | 1000 | 352 | 109 | 3.0 | |
| 51 | 1400 | | | 1.6 | |
| 53 | 1375 | 333 | 123 | | |
| 55 | 1750 | | | | |
| 57 | 1450 | 409 | 271 | | |
| 59 | 1350 | | | | |
| 61 | 1400 | 247 | 117 | 1.7 | |
| 63 | 1000 | | | | |
| 65 | 950 | 301 | 116 | | |
| 67 | 950 | | Finished experiment | | |

* glucose equivalent