GROWTH AND FERMENTATION FACTORS FOR DIFFERENT BREWERY YEASTS*

by

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Brewery fermentation is a complex process which involves both yeast growth and alcoholic fermentation, in addition to such other changes as are concerned with flavour. The reactions which occur may be studied by an analytical method, or a synthetic procedure may be used in which an attempt is made to reproduce the principal features of brewery fermentation in solutions of known composition. Eventually, it may be necessary to utilize fully both types of approach before enough is learned of the process to control it.

Recently, the problem has been attacked in our laboratories from the synthetic point of view. In an effort to study, separately at first, the factors which yeast requires for growth and fermentation, conditions and media were chosen such as would yield, in the former instance, maximal growth with little fermentation and then the reverse. Finally, to investigate those factors under conditions more closely related to actual brewery practice, conditions were set up intermediate between the two in which appreciable growth as well as fermentation occurs.

This work, here to be reported, hence consists of three phases:

1. A study of the bios requirements of various pure cultures of brewery yeasts for reproduction, growth being followed in a series of solutions at 30° C.

2. A study of initial rates of fermentation by pressed brewers' yeast in synthetic media as compared with wort.

3. Finally, an investigation of the combined processes of growth and fermentation under brewery fermentation conditions.

In the first study, the growth requirements of brewers' yeast cultures were studied under conditions designed to show the ability of the various organisms to synthesize the several bios or growth factors[1]. These tests were conducted at 30° C in shallow layers of media at a minute seeding rate. The extent of growth was measured turbidimetrically at the end of 40 hours. These studies disclosed that brewers' yeast strains differed as regards their bios requirements so that a number of different types could be identified. Types were shown to be relatively quite stable in respect to the particular factors required for growth. Thus it became possible to identify certain specific yeast cultures even after several years' use, in the brewery. Differences among brewery yeasts thus disclosed may be of value in the introduction of new cultures and in the maintenance of culture purity when, as is common brewing practice, yeast is used for many cycles.

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References p. 708.

In the second phase of our study dealing with fermentation rates, principal attention was paid to the fermentation kinetics as measured by the rate of CO_2 evolution during the first few hours of fermentation carried out at 30° C[2]. A medium was developed in which the initial rate of fermentation, using a relatively high yeast inoculum, is equal to that in beer wort. The various components of this synthetic solution were then investigated as to their individual influence on the rate, and their optimum concentrations were determined. Certain differences were found in the response by brewers' yeast to components of the synthetic solution as compared with the responses previously reported for bakers' yeast.

Normal brewery fermentations are conducted at low temperatures, relatively low seeding rates, and in deep vessels — circumstances which result in essentially anaerobic conditions in a short time. Although the principal reaction is alcoholic fermentation, a significant degree of yeast growth nevertheless takes place. For the successful production of beer, both processes appear essential. Therefore, in the third phase of our work, the synthetic medium was fermented at a temperature (12° C) comparable to brewery conditions and at a seeding rate corresponding to normal brewing practice. Under these conditions, using pressed brewery yeast, it was found that, unlike indications from the previous fermentations, the addition of a mixture of bios factors was needed before the synthetic solution could be made to ferment at a rate approaching that obtained with wort. On further examination, it was found that one of the bios factors, inositol, is the main factor for maximal fermentation rate under such conditions. It would seem that yeast, as taken from a normal brewery fermentation, has insufficient stores of inositol to permit it to grow in, and ferment, at an optimal rate, a synthetic solution deficient in this factor. Various aspects of the inositol requirement were studied, notably, the significance of this factor for two yeasts differing as to their need for inositol for growth. The effective inositol concentration also was determined and compared with the amount of this bios factor present in beer wort.

GROWTH FACTORS

Yeasts, in common with a great many other organisms, require more than pure sugar and salts for development of maximum growth rates. To obtain the growth and reproduction ordinarily observed in plant extracts such as beer wort, grape juice, etc., one or more of a group of substances known as bios factors (WILDIERS') must, it is now well known, be present. These factors are inositol, pantothenic acid, biotin, thiamin, and pyridoxine. Some yeasts, principally lactose fermenters[3], also are reputed to require nicotinic acid.

It has been shown by a number of investigators[4,5,6,7] that yeasts differ markedly in the number and combination of bios factors required. Cultures obtained from various national collections of type cultures showed marked differences in their bios requirements even among members of groups presumably closely related in their industrial uses (*e.g.*, distillers', brewers' yeasts, etc.) and which were so similar as to be indistinguishable by ordinary taxonomic techniques. The success of industrial fermentations depends greatly, it is generally believed, on the nature and the constitution of the particular strains of the microorganism in use. Hence, the potential practical significance of an increase in specificity of classification methods for the fermentation industries should be obvious.

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Because variation, mutation, and segregation have been reported as occurring in yeast cultures under one condition or another[8, 9] it was considered necessary to observe the constancy of the growth characteristics of the various cultures involved. In addition to the usual storage on agar slants, the cultures were subjected to various other treatments which were likely to be encountered in commercial practice and which might conceivably cause mutation to occur. The yeasts were then re-isolated (multiple isolates) and single cell isolates tested for their bios requirements under standardized conditions as described.

Methods and Apparatus

The medium is based on that used in microbiological vitamin assay techniques using yeast as the test organism[10]. The composition of this medium as finally adopted is shown in Table I. In practice, a series of more concentrated solutions are prepared containing a) the basal medium without bios factors, b) the five bios factors, and c), d), e) and f) bios solutions deficient in one or more of the factors. These solutions are preserved in the refrigerator under a preservative[11] composed of n-butyl-chloride (three volumes) and carbon tetrachloride (one volume).

TABLE I

COMPOSITION OF SYNTHETIC GROWTH MEDIUM

Dextrose, c.p. (anhydrous)	50	g	per 1 000 ml
Potassium phosphate (KH_2PO_4)	0.55	g	,, ,, ,,
Potassium chloride (KCl)	0.425	g	,, ,, ,,
Calcium chloride ($CaCl_2.2H_2O$)	0.125	g	,, ,, ,,
Magnesium sulphate ($MgSO_4.7H_2O$)	0.125	g	,, ,, ,,
Ferric chloride ($FeCl_3.6H_2O$)	2.5	mg	,, ,, ,,
Manganese sulphate ($MnSO_4.4H_2O$)	2.5	mg	,, ,, ,,
Potassium citrate buffer, p_H 5.5 (0.4 M)	50	ml	,, ,, ,,
Casein hydrolysate (vitamin-free) 8%, p_H 5.5	50	ml	,, ,, ,,
Inositol	25	mg	,, ,, ,,
Calcium pantothenate	2.5	mg	,, ,, ,,
Biotin	0.025	mg	,, ,, ,,
Thiamin hydrochloride	0.5	mg	,, ,, ,,
Pyridoxine hydrochloride	0.5	mg	,, ,, ,,

The inoculum is prepared from a fresh 24-hour growth (at 30° C) of the yeast on a malt-agar slant. A wire loop of yeast is transferred to 10 ml of sterile saline contained in a colorimeter tube. The concentration of yeast is determined and adjusted with additional saline or yeast to 1 mg per ml. An aliquot of the suspension is further diluted with sterile saline to a yeast concentration of 0.1 mg per ml. One ml of this final suspension is used to inoculate each flask.

The procedure employed was as follows: The various media were made to a volume of 9 ml in each of a series of flasks, plugged with cotton, sterilized in flowing steam for 20 minutes, cooled, and inoculated with 1 ml of a suspension containing 0.1 mg of the yeast under study.

The growth tests are conducted in a volume of 10 ml of medium contained in 125 ml Erlenmeyer flasks. The flasks are incubated at 30° C. When the measurement of yeast growth is used for vitamin assay or when the medium is contained in test tubes or smaller flasks, it is generally desirable to shake the vessels in order to incorporate an adequate supply of air. With the larger flasks employed in the present work, no difference due to shaking could be observed in parallel tests. Hence the flasks were not shaken during incubation in the present studies. Yeast growth is customarily estimated at 24 and 40 hours (duplicate flasks) but in the present report, only the 40-hour results have been considered.

The estimation of yeast growth is based upon the absorption of light in the EVELYN photoelectric colorimeter (660 filter). A calibration curve relating milligrams of moist yeast to absorption per cent was initially constructed using compressed bakers' yeast as a reference standard. The yeast solids equivalent corresponding to the calibration curve was determined for a number of different brewers' yeast cultures by first estimating the moist yeast content of a suspension from the curve and then determining the yeast solids content of an aliquot of the suspension by the A.S.B.C. method. The average of four such determinations was 28.98% (standard deviation = 0.72), *i.e.*, our estimation of the concentration of moist yeast is based on a yeast of approximately 29% solids, which is about average for compressed yeasts.

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EXPERIMENTAL

Yeast samples were obtained from a number of breweries and plated out on wort agar. Single colonies were selected at random and transferred to agar slants. They were replated to check purity and then stored at 5° C. A total of 58 cultures was collected in the first stage of this study. Of these, 53 represent lager (bottom) yeast cultures and the remaining 5, ale or top yeasts. All cultures were subjected to routine fermentation tests. In such tests the lager yeasts uniformly fermented melibiose whereas the ale yeasts did not.

When grown in the several media deficient in one or another of the bios factors, the various cultures showed significant differences in their ability to grow at a rate comparable to that shown in the so-called "complete" medium.

In the all factor medium, most of the cultures produced about 16 mg of moist yeast per ml in 40 hours. As a first approximation we considered that a yeast required a bios factor when growth in the absence of the factor was less than 50% of that shown by the same yeast in the all factor medium. On this basis, it is possible to group the cultures into five categories:

Those which require

1. biotin only
2. biotin and pantothenate
3. biotin and inositol
4. biotin, pantothenate and inositol
5. biotin, pantothenate, inositol and either thiamin or pyridoxine.

The growth of five different yeasts, representative of each of these types, is shown in Fig. 1, and the growth responses of the yeasts studied are summarized in Table II; here, the range of growth in the various media is given for all of the cultures in each group. It might be well to emphasize that the upper and lower figures for the range of growth in the table represent different yeasts and not results of replicate tests with the same culture. It will be observed that all of the yeasts require biotin, and some require either pantothenate or inositol or both. As regards thiamin and pyridoxine, the lager and ale yeasts show a sharp division. The lager yeasts were found to grow well when both thiamin and pyridoxine were simultaneously omitted from the medium. On the

TABLE II

BIOS REQUIREMENTS OF BREWERS' YEAST GROUPS
RANGE OF GROWTH — MG MOIST YEAST PER ML AT 40 HOURS (30° C)

Cultures	Required Factors	Growth Range	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B_1 & B_6 Omitted
14 Lager	Biot.	min	12.2	0.0	16.9	7.7	10.4
		max	15.0	0.5	13.2	14.4	17.2
23 Lager	Biot., Panto.	min	11.4	0.1	0.2	7.6	12.6
		max	15.0	0.6	7.4	15.0	16.0
4 Lager	Biot., Inos.	min	12.8	0.1	8.5	1.3	12.0
		max	16.0	0.3	16.0	6.4	16.0
12 Lager	Biot., Panto., Inos.	min	12.2	0.2	0.0	0.6	10.8
		max	15.0	0.8	4.8	6.1	15.0
5 Ale	Biot., Panto., Inos., B_1 or B_6	min	12.1	0.1	0.0	0.0	1.0
		max	13.6	0.2	0.1	1.0	5.5

References p. 708.

other hand, the ale yeasts required either thiamin or pyridoxine and grew very poorly when both were omitted. This curious apparent equivalence of pyridoxine and thiamin has been previously reported by SCHULTZ *et al.*[4] in studies with a variety of bakers' yeasts.

Since in the case of lager yeasts, each requires biotin and none requires either thiamin or pyridoxine, there are four possible types, *i.e.*, lager strains which in addition to biotin:

1. require no other factor
2. require pantothenate
3. require inositol
4. require pantothenate and inositol.

As may be seen, representatives of each of these groups have been found among lager yeasts.

The reproducibility of the typing method and the stability of the bios types were studied in several ways. In order to determine the influence of extended storage, for example, culture maintenance on agar slants at 5° C, the cultures so maintained were periodically examined or tested for their bios responses. The results obtained with a group of representative yeasts after storage on agar slants for periods ranging from 97 to 302 days are shown in Table III, from which it may be seen that the initial characteristics were maintained without marked alteration. In the case of culture 6C56, it

TABLE III

INFLUENCE OF EXTENDED STORAGE AT 5° C (AGAR SLANTS) ON BIOS REQUIREMENTS OF BREWERS' YEASTS
GROWTH AT 40 HOURS (30° C) — MG MOIST YEAST PER ML

Culture	Required Factors	Storage (days)	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B_1 & B_6 Omitted
Lager 7C8	Biot.	0	13.6	0.3	11.4	12.2	13.6
		188	14.4	0.2	9.2	12.8	14.4
Lager 6C13	Biot., Panto.	0	16.8	0.2	0.2	13.7	16.1
		302	14.4	0.2	0.8	13.2	14.4
Lager 7C30	Biot., Inos.	0	16.0	0.2	16.0	1.3	16.0
		178	16.0	0.2	16.0	0.8	16.0
Lager 6C56	Biot., Panto., Inos.	0	11.7	0.2	0.1	4.4	11.7
		226	13.2	0.2	0.2	0.8	12.8
Ale 6C3	{Biot., Panto., Inos., B_1 or B_6}	0	12.1	0.1	0.1	0.3	1.0
		97	14.0	0.0	0.0	0.1	2.4
Bakers' 6C1	Biot., Panto., B_1 or B_6	0	13.4	0.3	0.1	8.4	0.1
		97	14.0	0.6	0.0	8.1	0.6

may be observed that the ability to grow slightly without inositol appears to have been lost or weakened after extended storage.

As a further test of the constancy, and incidentally the reproducibility, of bios type, a number of yeasts were subjected to daily serial transfer, week ends excepted, *i.e.*, the culture was transferred daily from agar slant to agar slant, being incubated between transfers at 30° C. Under these conditions, the yeast is forced to undergo rapid, extensive multiplication at relatively high temperatures for a considerable period of time.

The results of this study are shown in Table IV; the number of serial transfers ranged from 13 to 32. It may be seen that again the basic bios characteristics have been

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TABLE IV

INFLUENCE OF DAILY SERIAL TRANSFER ON BIOS REQUIREMENTS
GROWTH AT 40 HOURS (30° C) — MG MOIST YEAST PER ML

Culture	Required Factors	Number of Transfers	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B_1 & B_6 Omitted
Lager 7C8	Biot.	0	13.6	0.3	11.4	12.2	13.6
		28	14.4	0.0	14.0	13.2	14.4
Lager 6C13	Biot., Panto.	0	16.8	0.2	0.2	13.7	16.1
		32	11.0	0.2	0.2	8.6	11.4
Lager 6C56	Biot., Panto., Inos.	0	10.6	0.2	0.2	2.3	10.8
		13	12.2	0.2	0.0	0.3	12.2
Ale 6C3	Biot., Panto., Inos., B_1 or B_6	0	12.1	0.1	0.1	0.3	1.0
		32	14.4	0.0	0.0	0.2	3.4
Bakers' 6C1	Biot., Panto., Inos., B_1 or B_6	0	13.4	0.3	0.1	8.4	0.1
		13	13.6	0.6	0.0	6.2	0.4

maintained, although in several instances there appears to have been a weakening of the culture. This weakening appears in a reduction of the extent of growth on the all factor medium and also a reduction of the extent of growth on one or more of the deficient media.

A test to disclose possible mutation was the typing of yeasts before and after fermentation of hopped brewery wort at 10° C (simulating brewery conditions). This test was conducted as follows: The culture was brought up in increasing volumes of brewery wort and finally seeded into two liters of hopped brewery wort at 10° C where it was allowed to end-ferment (10 days) and then permitted to rest under the beer for another five days, following which it was plated out and six colonies were selected at random from each of the plates for subculture. These cultures were then tested for their bios type, with results which may be seen in Table V, where the results obtained initially on the culture and the results obtained on the first two of the isolates are compared. It will be noted that the cultures were all recovered with very little change.

TABLE V

RECOVERY OF BIOS TYPES AFTER FERMENTATION OF BREWERY WORT AT 10° C
GROWTH AT 40 HOURS (30° C) — MG MOIST YEAST PER ML

Culture	Required Factors	Growth	All Factors	Biotin Omitted	Panto. Omitted	Inos. Omitted	B_1 & B_6 Omitted
Lager 7C35	Biot.	Init.	14.4	0.2	12.2	13.2	14.4
		Final*	14.4	0.2	13.2	12.8	15.0
		Final*	14.4	0.2	12.6	12.8	15.0
Lager 7C29	Biot., Panto.	Init.	14.0	0.2	0.2	13.6	14.0
		Final*	14.4	0.2	0.2	13.6	14.4
		Final*	13.2	0.3	0.2	13.2	14.0
Lager 7C30	Biot., Inos.	Init.	16.0	0.2	16.0	1.3	16.0
		Final*	17.2	0.2	17.2	2.0	18.8
		Final	17.6	0.2	16.4	1.8	18.8
Lager 7C6	Biot., Panto., Inos.	Init.	12.6	0.4	0.4	1.3	12.6
		Final*	13.2	0.3	0.4	2.0	14.4
		Final*	13.2	0.3	0.3	3.4	13.2

* First two isolates of a series of six or more.

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Ever since the time of PASTEUR AND HANSEN, the importance of pure yeast culture in the art of brewing beer has been everywhere appreciated by brewers. PASTEUR first demonstrated the importance of having the yeast free from harmful bacteria and then HANSEN showed the value of specific pure yeast strains. Many a successful brewer has justifiably felt that the desired characteristics of his beer were largely due to the special properties of his particular strain. It is, therefore, perhaps not surprising that we find marked differences in the bios types of the yeasts employed in the various breweries, denoting not only different origins but also the possible effect of continued selection. Although the tests which we have thus far made do not indicate significant mutation, it is quite possible that over longer periods of time the process of mutation may have been responsible for the origin of differences in strains of brewers' yeasts.

The long range objective of research in this field is, of course, to learn how and why these various yeasts differ, one from the other, in industrial processes. In other words, it is hoped better to define the differences between them and, if possible, to determine the biochemical basis for the differences. A logical part of such a program is the study of fermentation rates in synthetic solutions, which forms the next phase of the work here presented.

FERMENTATION FACTORS

The principal reaction brought about by yeast in brewery wort is alcoholic fermentation, although a significant growth of yeast is necessary in each fermentation cycle if only to obtain sufficient pitching yeast for the succeeding cycle. Although, classically, fermentation has been defined as representing cell metabolism in the absence of oxygen, it is very difficult, perhaps impossible, experimentally to separate fermentation from growth when it is desired to study each activity separately. By limiting the supply of oxygen and providing elevated concentrations of yeast and sugar, one may fairly successfully observe reactions essentially fermentative in character. Reactions observed under such conditions can be described as assimilative fermentation[5].

As carried out by us, one gram of moist yeast is suspended in 100 ml of medium, the mixture is shaken at 30° C, and the rate of fermentation is observed by measuring the volume of gas evolved at suitable intervals. In the course of three hours, one part of yeast converts six to eight parts of sugar. This fermentation is accompanied by a limited increase in yeast dry weight (about 20%). Thus, a predominantly fermentative reaction is established without extreme departure from the normal environment of beer yeast such as is produced by the addition of metabolic poisons like sodium azide.

Utilizing these conditions, an experimental medium was developed which incorporated factors known to favour fermentation by living yeast. The rate of fermentation in this synthetic medium was compared with the rate in beer wort. With suitable changes in composition, it was found that the rate of fermentation in such a medium (corresponding to a drop in apparent extract of about 2° Plato) was equal or superior to that observed in beer wort. Each of the components of the medium was separately investigated as to its necessity, and the concentration of each corresponding to optimal fermentation rate was determined. The composition of the final medium is given in Table VI.

The most noticeable effects were produced by the omission of NH_4^+, $PO_4^{\equiv}$, Mg^{++}, $SO_4^{=}$, and K^+. The absence of NH_4^+ and $PO_4^{\equiv}$ caused a marked reduction in fermentation

References p. 708.

TABLE VI

SYNTHETIC FERMENTATION MEDIUM

Dextrose	50	g	per 1000 ml
Ammonium chloride (NH_4Cl)	1.87	g	,, ,, ,,
Sodium phosphate ($NaH_2PO_4.H_2O$)	2.48	g	,, ,, ,,
Potassium chloride (KCl)	1.34	g	,, ,, ,,
Magnesium sulphate ($MgSO_4.7H_2O$)	0.800	g	,, ,, ,,
Calcium chloride ($CaCl_2$)	0.333	g	,, ,, ,,
Sodium succinate buffer, pH 4.9 (M/3)	150	ml	,, ,, ,,
Casein hydrolysate (vitamin-free) 8%, pH 5.5	25	ml	,, ,, ,,
Thiamin hydrochloride	0.800	mg	,, ,, ,,
Pyridoxine hydrochloride	0.800	mg	,, ,, ,,
Nicotinic acid	8.00	mg	,, ,, ,,

rate. Mg^{++}, $SO_4^=$, and K^+ were also necessary for the maximal rate of fermentation, although to a lesser extent. In order to accomplish the single omission of various ions, appropriate corresponding Na^+ or Cl^- salts were substituted, *i.e.*, $MgCl_2$ replaced $MgSO_4$, in order to observe the effect of omission of $SO_4^=$. Similarly, Na_2SO_4 replaced $MgSO_4$ in order to study the effect of absence of Mg^{++}.

In the investigation of the influence of the various ions, it was observed that a rather small excess of Mg^{++} over that necessary caused a marked depression in fermentation rate. The addition of a small quantity of $CaCl_2$ counteracted this inhibition and augmented the stimulating effect of low concentrations of Mg^{++}. For this reason, $CaCl_2$ was incorporated in the basic medium.

The experimental omission of thiamin, pyridoxine, and nicotinic acid had relatively little effect; nevertheless, these factors were included in the medium because, in the case of bakers' yeast, the importance of these factors had been established by other workers[12]. Other bios factors, biotin, calcium pantothenate, and inositol, were experimentally added without noticeable effect, most probably due to the relatively small extent of growth involved in these tests. Hence, these factors were not incorporated in the basic medium. As will be described later, when conditions were so adjusted as to favour a significant proportion of yeast growth concomitantly with fermentation, the last named factor, inositol, was found to play a very important role.

In Table VII are shown the results of an experiment in which the fermentation rate of beer wort under the above conditions is compared with fermentation in the synthetic medium as finally evolved (Table VI). Since the fermentable sugars of beer wort consist of both maltose and dextrose, experiments were made first with these separately as the sole sugars in the synthetic medium and then with a mixture of four parts of maltose and one part of dextrose. The latter mixture corresponds to the proportions we have found in samples of brewery wort by a method based upon selective fermentation by different yeast species[13]. It will be observed from Table VII that the rate of fermentation in the synthetic medium containing 4% of maltose and 1% of dextrose is quite equal to that observed in beer wort. In the synthetic medium in which maltose is the only fermentable sugar, the fermentation rate is considerably lower than that of wort, whereas when dextrose is the sole sugar, the rate of fermentation soon exceeds the wort rate.

This synthetic fermentation medium is obviously not in any sense a synthetic beer wort but it would appear that it contains the substances, or their physiological equivalents, necessary for a rapid initiation of fermentation at 30° C. A medium of this type might be employed as a reliable and reproducible reference standard for ascer-

References p. 708.

TABLE VII

RATES OF FERMENTATION IN SYNTHETIC MEDIUM AND BEER WORT

	Sugar	Gas evolved			
	Per cent w/v	60 min	120 min	180 min	240 min
Beer wort	—	68	159	285	433
Synthetic	Maltose 5	33	83	176	313
Synthetic	Dextrose 5	57	146	294	484
Synthetic	Maltose 4 Dextrose 1	67	160	287	443

taining possible deficiencies in beer worts or other fermentation media. A method based on this principle would be similar to the microbiological assay methods widely employed for vitamins, amino acids, etc. Conversely, such a method may be used to assess quickly the vitality and characteristics of pitching yeasts in brewery operations.

GROWTH AND FERMENTATION IN EXPERIMENTAL MEDIA

Having now separately studied first yeast growth and then fermentation in synthetic solutions at 30° C, it was thought desirable to extend the investigation to conditions more closely approaching those obtaining in brewery fermentation. Principal differences here are to be found in pitching rate and temperature. In order to effectively emphasize growth and fermentation in the respective studies, the growth tests had been made with a very low pitching rate (moist yeast concentration 0.01 mg per ml or 0.0026 lb per bbl). On the other hand, in the case of the fermentation tests, a pitching rate was employed equivalent to about 2.5 pounds per barrel or 10 mg per ml. An average normal pitching rate is about 0.5 lb per bbl in terms of moist or pressed yeast. This is the pitching rate which was used in the final attenuation experiments. This rate is equivalent to 1.94 mg per ml. An experimental temperature of 12° C was considered to be close to brewery fermenting temperatures. These conditions may be seen to involve a degree of growth not occurring with a pitching rate five times as great, while fermentation, at the same time, is considerably slowed both by the lower temperatures and reduced pitching rate. With these conditions as to pitching rate and temperature and using the synthetic fermentation medium shown in Table VI, the rate of attenuation was compared to that of wort. Here, the synthetic medium is found to be markedly inferior, a difference which was heightened when pitching rates were lowered further to half-normal values, *i.e.*, 1 mg per ml. Evidently, under such conditions the medium is deficient in some factor or factors present in beer wort. Experiments showed that addition to the synthetic medium of either yeast extract or a mixture of the bios factors increased the attenuation rate to that observed in wort. Inositol was disclosed as the most important of the bios factors responsible. This effect of inositol was then studied with two yeasts of differing bios types and the minimum effective concentration of inositol determined for comparison with the concentrations normally occurring in beer wort.

References p. 708.

METHODS AND APPARATUS

Fermentations were conducted in a volume of 500 ml of media contained in one-liter pyrex bottles equipped with water seals. The bottles were incubated in a constant-temperature chamber at 12° C.

The Synthetic Medium employed was based on that shown in Table VI with the modifications noted, *i.e.*, the dextrose content was increased to 70 g per liter, the succinate buffer was reduced to 100 ml per liter, and additional bios factors were added as required.

The Wort was lager cooler wort obtained through the courtesy of a local brewery, brought to the laboratory in glass containers, filtered and boiled for three minutes in cotton-plugged flasks.

The Yeast was washed, compressed brewers' yeast, obtained from two local breweries, and designated for convenience as yeast A and B. Both of these breweries employ pure culture apparatus. The yeasts were typed as to growth requirements by the methods already described with results showing that:

Yeast A is similar to culture 7C43 (see Fig. 1) in that it requires biotin, pantothenate and inositol.

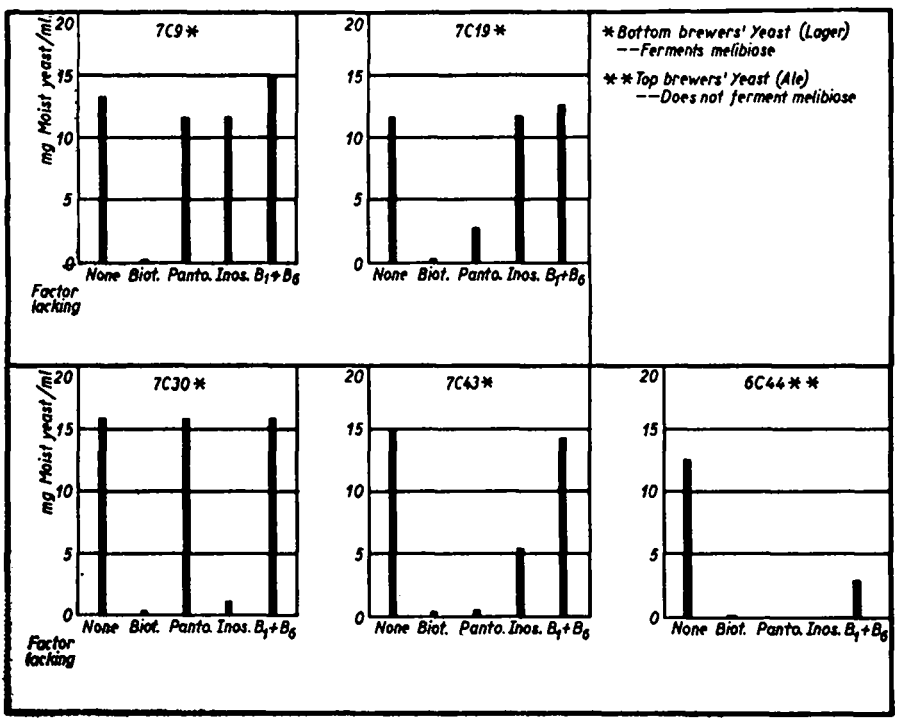

Fig. 1

Yeast B is similar to culture 7C9 (see Fig. 1) in that it requires only biotin.

The Rate of Attenuation was followed by daily measurements of the specific gravity. Fermentations were not agitated after the first 24 hours. One ml samples were removed each day and, after degassing, the specific gravity was determined in a specific gravity gradient tube[10].

The Reagents were, in all cases, the best chemically pure grades available. The yeast extract was obtained from the DIFCO LABORATORIES INC.

EXPERIMENTAL

For this phase of the work, some modifications of the medium of Table VI were

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found desirable. The dextrose content was increased to 7%. Some modification was made in the buffer content. In Fig. 2 are shown the rates of attenuation of this medium (with and without bios factors) as compared with that of beer wort. Preliminary experiments had shown a faster rate of attenuation of wort; consequently this experiment included tests in which yeast extract or a mixture of bios factors was added to the basal synthetic medium.

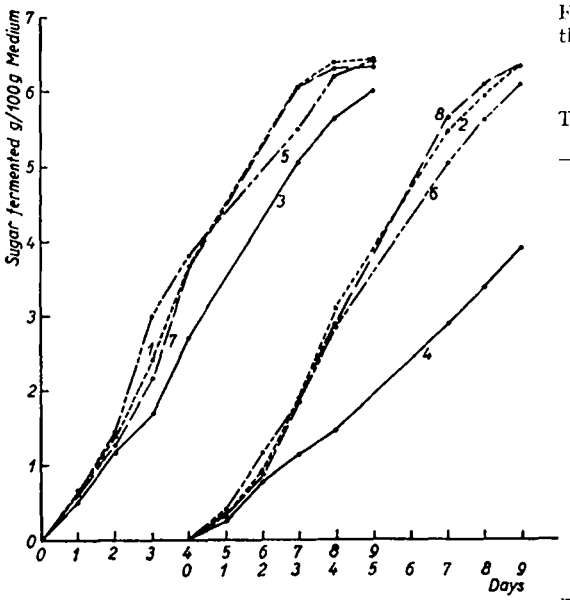

Fig. 2. Rate of Attenuation in Wort and Synthetic Medium Supplemented with Yeast Extract or Additional Bios Factors

Test	Medium (500 ml)	Pitching Rate Pressed Yeast mg/ml
1	Beer wort, 12° P	1.94
2	Beer wort, 12° P	0.97
3	* Synthetic medium (7% dextrose)	1.94
4	* Synthetic medium (7% dextrose)	0.97
5	* Synthetic medium (7% dextrose) plus 1.00 g yeast extract	1.94
6	* Synthetic medium (7% dextrose) plus 1.00 g yeast extract	0.97
7	** Synthetic medium (7% dextrose) plus six bios factors	1.94
8	** Synthetic medium (7% dextrose) plus six bios factors	0.97

* No bios factors

** Biotin, 0.25 mg; Calcium pantothenate, 5.00 mg; Inositol, 5.00 mg; Thiamin, 0.40 mg; Pyridoxine 0.40 mg; Nicotinic acid 4.00 mg.

From the course of the fermentations as shown in the graph, it can be seen that the addition of either yeast extract or the bios mixture, (biotin, pantothenate, thiamin, pyridoxine, nicotinic acid and inositol) causes an increase in attenuation rate so that the supplemented solutions are equal to wort. In Fig. 2, the extent of fermentation is indicated in terms of sugar fermented.

It is apparent that the necessity for supplementation of the synthetic medium is greater at the lower pitching rate. This, no doubt, is a reflection of the additional yeast growth required at lower pitching rates if a normal yeast crop and consequent normal rate of fermentation are to be developed. Since we were primarily interested in comparing beer wort and the synthetic medium, the lower pitching rate was employed to emphasize such differences in the experiments which follow.

The Effect of Inositol

Since it was apparent that the factor or factors responsible for the increased rate of attenuation of the supplemented medium could be found in the bios mixture, a series, of tests were made in which the bios factors were singly omitted. As a matter of interest, the need for those bios factors originally present in the basal medium, *i.e.*, thiamin pyridoxine and nicotinic acid, was also studied in this series of tests. The results are

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shown in Table VIII, from which it is apparent that inositol is the dominant factor for fermentation under these conditions. The omission of inositol alone caused a reduction in rate as marked as the omission of all of the bios factors.

TABLE VIII

INFLUENCE ON ATTENUATION RATE OF OMISSION OF BIOS FACTORS FROM SYNTHETIC MEDIUM* (PITCHING RATE = 0.97 MG PRESSED YEAST PER ML TEMP. = 12° C)

Bios Factor Omitted	Per Cent Sugar Fermented G sugar/100 g medium	
	3 days	6 days
None	2.9	5.9
Inositol	1.5	3.6
Pantothenate	2.6	5.8
Biotin	2.7	5.8
Thiamin	2.8	5.5
Pyridoxine	2.8	5.9
Nicotinic acid	2.9	5.7
All bios factors	1.8	3.8

* Synthetic medium as shown in Table VI with the following changes: Dextrose, 70 g; Sodium succinate buffer, 100 ml; Biotin, 0.50 mg; Calcium pantothenate and Inositol, 10.0 mg per liter each.

Different Yeast Types

It is apparent that the importance of inositol for normal attenuation in synthetic media might hinge on the specific bios type of the brewers' yeast culture employed, *i.e.*, an inositol effect might be expected by those yeasts which cannot synthesize this bios factor but might not be expected for others. Of the two pressed brewery yeasts available for this study, yeast A required biotin, inositol, and pantothenate, while

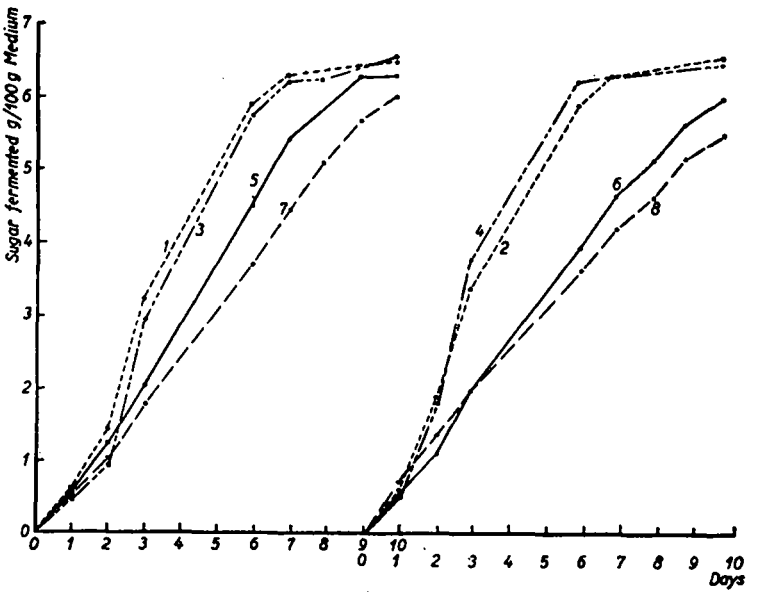

Fig. 3. The influence of inositol and pantothenate on the rate of attenuation by two different yeasts

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yeast B required only biotin. In the series of experiments represented in Fig. 3, the two yeasts were compared. The rate of fermentation by each yeast in each of four solutions is here depicted. One solution contained all six bios factors (Test 1 and 2), one contained all but pantothenate (Test 3 and 4), one contained all but inositol (Test 5 and 6), and one contained no bios factors whatsoever (Test 7 and 8).

Test No.	Medium (500 ml)	Yeast (0.97 mg/ml)
1	*Synthetic medium with all bios factors	A
2	Synthetic medium with all bios factors	B
3	Synthetic medium with all factors except pantothenate	A
4	Synthetic medium with all factors except pantothenate	B
5	Synthetic medium with all factors except inositol	A
6	Synthetic medium with all factors except inositol	B
7	Synthetic medium without bios factors	A
8	Synthetic medium without bios factors	B

* Synthetic medium with all bios factors as shown in Table VIII.

As the results show, inositol is necessary in the case of both yeasts and the lack of pantothenate is without significant effect. Thus the difference in bios type is not reflected under these experimental conditions. It should not be necessary to dwell at length on the wide difference between the conditions of this test and the conditions employed in the bios typing tests. It is probably sufficient to point out that, in the present tests, there is no more than an eight-fold multiplication of the yeast, whereas in the bios typing method, multiplication of the yeast inoculum may range from 500 to 1600 times. The difference in response to an inositol deficient medium is probably related to the inability of yeast to store inositol in excess of needs for multiplication and in the case of a yeast which can synthesize inositol, also inability to do so with sufficient rapidity under these experimental conditions.

The Specific Gravity of the Synthetic Medium. As previously noted, the gravity of the synthetic medium is considerably lower than that of wort both initially and during the course of fermentation. This is because the non-fermentable dextrins of beer wort have no counterpart in the synthetic medium. It was considered of interest experimentally to adjust the density of the synthetic medium to bring it in closer approximation to wort in this respect. Since well defined dextrins were not available, it was thought that lactose, a non-fermentable carbohydrate, might serve. Accordingly, 32.2 g per liter of lactose were incorporated in a synthetic medium. As shown in Fig. 4, the attenuation of this medium containing all of the bios factors was found to compare favorably with that observed in wort. Actually, the rate of attenuation of the synthetic medium is greater than that observed in wort. This is probably due to the fact that the synthetic medium contains dextrose as the only fermentable sugar.

Influence of Other Bios Factors. In order to determine whether inositol is the only bios factor required to reproduce the attenuation rate of wort, experiments shown in Fig. 4 are of interest. One is a fermentation of a synthetic medium to which none of the bios factors was added, and the other the fermentation of a medium to which only inositol was added. As may be seen, while inositol alone produces a pronounced stimulation, the other bios factors are also essential for development of a maximal rate.

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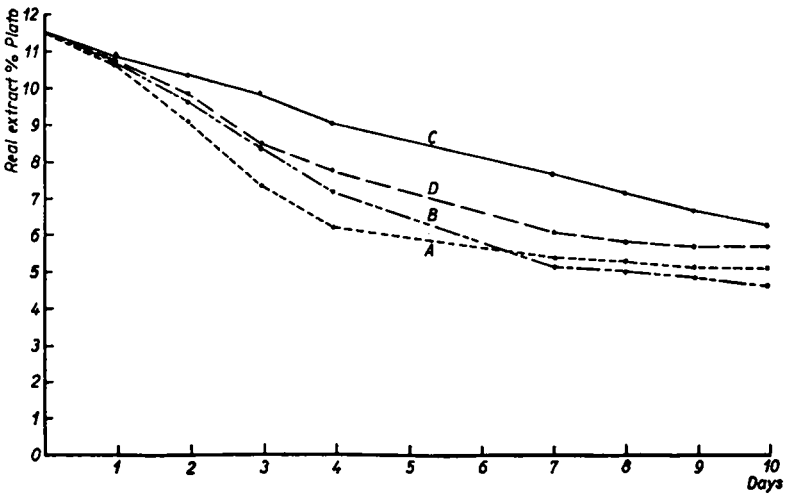

Fig. 4. Rate of attenuation of wort compared with synthetic media (with Plato adjusted by addition of lactose), yeast B.

Curve A. Synthetic medium with all bios factors as in Table VIII plus 16.1 g lactose per 500 ml.
Curve B. Brewery wort.
Curve C. Synthetic medium as in A but without bios factors.
Curve D. Synthetic medium as in A but with inositol as only bios factor.

Effective Inositol Concentrations. It is natural to inquire whether inositol might ever become a limiting factor in actual beer wort fermentation in the brewery. To answer this question, a series of tests were made with graded concentrations of inositol. It was found that a concentration of 1.5 mg of inositol per liter was adequate for the maximal effect. Several samples of brewery wort, separately analysed for available inositol by a yeast microbiological assay method[10], gave values ranging from 35 to 50 mg of inositol per liter. Based on such values, it would appear that an inositol deficiency is not likely to occur in normal brewing practice. However, further investigation may well reveal the existence, at least under some circumstances (*e.g.*, due to varietal differences in barley or processing conditions) of antimetabolite substances acting as anti-vitamins. A close similarity between the structure of a recently introduced insecticide, hexachlorocyclohexane (gamma isomer) and inositol points to at least one possible relationship of this kind. Some tests already conducted have shown such an anti-inositol effect in the case of this gamma isomer.

DISCUSSION

Our observations in the third phase of the work conducted with conditions more closely approaching those of brewery fermentation represent only a beginning. The fact that inositol, in these experiments, appears to play a dominant role in attenuation, and the observation that this behaviour is not directly correlated with a growth requirement for inositol merit some consideration and explanation, if possible. One explanation that suggests itself is that inositol may be more than simply a growth factor. It may, in fact, play a specific role in fermentation under the experimental conditions employed. Such a role for inositol has not been previously reported, and there is a lack of any contributory evidence which would, at present, support such a concept.

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The explanation which seems more likely to be correct, in the present state of our knowledge, hinges on the relative rates of growth occurring in the three experimental yeast environments employed. One environment heavily emphasized growth, resulting in a multiplication of 500 to 1600 times, another minimized growth so that the yeast dry matter increased by only one-fifth of its initial weight during the tests. Furthermore, in the first case, only yeast growth was measured; and in the second case, only alcoholic fermentation. Finally, in the attenuation experiments, the number of multiplications of the yeast is intermediate, of the order of four to eight, and our measurements (of specific gravity) were essentially measurements of the rate of alcoholic fermentation. Under these conditions, the rate of attenuation should reflect both the increase in the number of yeast cells and the fermentative activity of the number of cells present at each interval. Furthermore, the resulting curves are very sensitive to relatively small deviations in either factor, *i.e.*, cell number or fermentation intensity, because the reading records the *cumulative* effect, over a period of time, of changes in the sugar substrate. It is apparent from the results that unless the yeast receives an exogenous source of inositol, it cannot, under these conditions, manufacture this compound rapidly enough to coordinate with its other metabolic activities even though it has the capacity to do so eventually.

It has been shown that yeasts are able to absorb or otherwise retain large quantities of thiamin, one of the other bios factors. There is evidence that the very high amounts of this bios factor accumulated by the yeast represent a substantial excess over its normal metabolic requirements. The biotin content of yeast also has been shown to vary rather widely, although not to the same extreme degree as thiamin. It is probable that inositol is in a different class in this respect. Yeast may not be able to store up inositol in quantities more than enough for its functional requirements. In general, inositol differs from the other bios factors in other ways. It forms a rather large proportion of yeast dry matter, 0.5%. It may also differ from the other bios factors insofar as it may be a structural element, *i.e.*, a compound of the phospholipid lipositol rather than a catalytic factor such as a coenzyme postulated for all of the other five bios factors.

Although the other bios factors do not seem to play a role in our test in which the attenuation of synthetic solutions was followed when the yeast was taken from the bios-rich beer wort and pitched in the synthetic solution, it is very likely that successive fermentation cycles of these yeasts in synthetic media would disclose a need for the other bios factors in accordance with the requirements found in this bios typing procedure.

Even though two different brewers' yeasts were employed in the present study, it does not seem justifiable to generalize regarding the influence of inositol until other yeasts have been examined; yeasts which have been produced under different conditions and which originate from other cultures. Another factor which might be mentioned in the above connection is that bios typing interpretations are based upon a growth over a prolonged period (41 hours). In a shorter period (24 hours), virtually all of the yeasts show a stimulation of growth due to each of the bios factors.

With respect to the fermentation studies at 30° C in the fermentometer, this has been found adaptable to practical uses. Measurements of fermentative activity of liquid brewery yeasts may be made by this technique. In this way, it is possible to assess the vitality and characteristics of pitching yeasts in a short time. This has, in fact, been done with considerable success.

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Although these investigations admittedly were far from complete, there has already developed a most useful application of the bios typing technique. It is widely recognized that standard methods of yeast classification are not wholly satisfactory. Usual tests are not adequately sensitive to the differences known to exist between strains of yeast. All lager yeasts, regardless of suitability for brewing, would probably be classified as *S. carlsbergensis*, STELLING-DEKKER. By means of the bios typing technique, it is possible to separate strains of yeast otherwise considered to be equivalent by previous methods of classification. This faculty has, in fact, already been put to practical use in several instances.

We have, for example, been able to determine that a yeast culture, introduced into a particular brewery 10 years earlier, has remained constant and essentially pure. This was established by comparison of a stock culture which had been maintained in the laboratory with yeast from the brewery. In two other cases, it was established in this way that the original culture was no longer present, in one instance apparently due to adventitious contamination and in another, to a change of culture not at first reported to the investigator conducting the bios typing of the yeast.

The bios typing technique would be of even greater utility were it true that each particular bios type was uniquely associated with a yeast strain of singular characteristics. Unfortunately, this does not appear to be the case, no such invariable correlation having been observed. There are indications, however, that a practical correlation may be found; for example, one useful lager culture was encountered which required only biotin for growth; another culture, also useful, required biotin, pantothenate and inositol. It so happened that the first culture was characterized as being dustier than the second. When these two cultures were used in a brewery, one gradually replacing the other, it was possible to follow the purity of the incoming culture, thereby enabling correlation of practical observations with the specific yeast. It is expected that applications of this kind will be multiplied as further experience is obtained.

SUMMARY

In this investigation, an attempt has been made to study growth and fermentation of brewery yeasts, as far as possible, in solutions of known composition. Growth and fermentation were first studied separately at a temperature of 30° C and with conditions adjusted as to emphasize first growth under one set of conditions and then alcoholic fermentation under another. Finally, the combined process as it occurs in brewery fermentation was studied at lower temperatures, also using solutions of known composition. In the growth studies, it was found that lager yeasts of different strains showed marked differences in their growth requirements. These growth requirements were found to be relatively stable and could be used to identify certain specific strains of yeast. In the fermentation studies, yeast nutrients were combined to produce a medium in which the fermentation rate was equal to that obtainable with beer wort. In the third phase of the work, this medium was employed under conditions of brewery fermentation such that both growth and fermentation occurred and, in this instance, it was found that inositol played a very important role in determining the rate of attenuation. The significance of this finding was further investigated with different brewery yeasts.

RÉSUMÉ

Dans ces recherches nous nous sommes appliqués à étudier la végétation et la fermentation de levures de brasserie, autant que possible dans des solutions de composition connue. Au début la végétation et la fermentation furent étudiées séparément à une température de 30° C dans des conditions choisies de façon à accentuer d'abord la première végétation, puis la fermentation alcoolique. Finalement le procédé combiné se produisant au cours de la fermentation en brasserie fut étudié à une

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température moins élevée, également dans des solutions de composition connue. Au cours des études de végétation on a trouvé que les diverses variétés de levure de fermentation basse sont nettement différentes en ce qui concerne leurs besoins végétatifs. Il a été établi que ces besoins sont relativement stables et qu'ils sont utilisables pour l'identification de quelques variétés de levure spécifiées. Au cours des études de fermentation, des substances nutritives ont été combinées afin de produire un milieu dans lequel la vitesse de fermentation était égale à celle constatée dans le moût de brasserie. Dans la troisième phase du travail ce milieu fut employé dans des conditions de fermentation en brasserie, telles que la végétation et la fermentation se produisaient; on a trouvé dans ce cas que l'inositol joue un rôle très important en déterminant la vitesse d'atténuation. L'importance de cette découverte a été examinée de plus près en utilisant différentes levures de brasserie.

ZUSAMMENFASSUNG

Bei dieser Untersuchung wurde ein Versuch gemacht, das Wachstum und die Gärung von Brauereihefen soviel als möglich in Lösungen bekannter Zusammensetzung zu studieren. Wachstum und Gärung wurden zuerst einzeln bei einer Temperatur von 30° C studiert, einerseits unter Bedingungen, welche das erste Wachstum speziell förderten, anderseits unter solchen, bei denen der Nachdruck auf die Gärung gelegt wurde. Schliesslich wurde der kombinierte Prozess, so wie man diesem bei der Gärung in der Brauerei begegnet, bei niedrigeren Temperaturen studiert, wobei ebenfalls Lösungen bekannter Zusammensetzung gebraucht wurden. Bei den Wachstumsstudien wurde gefunden, dass untergärige Hefen verschiedener Rassen deutliche Unterschiede ihrer Wachstumsbedürfnisse zeigten. Diese Wachstumsbedürfnisse zeigten sich relativ stabil und konnten zur Identifizierung gewisser bestimmter Heferassen herangezogen werden. Bei den Gärungsstudien wurden Hefenährstoffe zu einem Medium kombiniert, in welchem die Vergärungsgeschwindigkeit derjenigen der Bierwürze gleichkam. In der dritten Phase der Arbeit wurde dieses Medium unter den Umständen der Brauereigärung verwendet, in der Weise, dass sowohl Wachstum als Vergärung auftraten und in diesem Fall wurde gefunden, dass das Inositol eine sehr wichtige Rolle spielt, indem es die Vergärungsgeschwindigkeit entscheidend bestimmt. Die Bedeutung dieser Entdeckung wurde weiter an verschiedenen Brauereihefen untersucht.

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