PHYSIOLOGICAL CONSEQUENCES OF POLYPLOIDY IN YEASTS

I. FERMENTATION CHARACTERISTICS OF A DIPLOID BREWERY YEAST AND ITS AUTOTETRAPLOID

by

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INTRODUCTION

Induction of polyploidy has been shown to be an important method for the improvement of economically important plants[1-6]. From an analogy it can be expected that similar methods should be applicable in the case of microorganisms of industrial importance, like yeasts. The lack of actual demonstration of the chromosomal mechanism in microorganisms has stood in the way of any fruitful research in this direction.

The few early instances of polyploidy in microorganisms are those reported by SORGEL[7] and WHELDEN[8]. Conflicting reports[9,10] have been published regarding an improvement in the antibiotic producing capacity of a strain of *P. notatum* through successive duplication of its chromosome complement.

The alterations in the metabolic activity of yeast cells under the influence of various physical[11-18] and chemical[19-33] mutagenic agents have not been analysed on a rational basis. Except in a few cases[28-33] sufficient information is not available as to the nature of the genetical changes undergone by the organisms as a result of such treatments. A clear evaluation of the effects necessitates the isolation of the new cell types, if any, obtained after the treatment with the polyploidising agent, and a comparative study of the cytology and physiology of the mutants with those of the parent strain. In the case of yeasts the basic physiological character for such a comparison should be their fermentative behaviour.

Definite criteria are not available to explain the remarkable divergences in the fermentative abilities of yeast types belonging apparently to the same species, although various attempts have been made from time to time. The futility of relying on the morphological and giant colony characteristics for such distinctions has been amply proved by the works of HANSEN[35] and LINDNER[36]. In spite of the agreement in the observed reciprocal relationship between the attenuating power and the flocculation rates of the various types of yeasts like the Saaz and the Frohberg, there is good reason to believe that such differences in the physical behaviour during fermentation may have little to do with the racial characteristics of the strains[37,38]. The characterisation of the low and the high attenuative strains on the basis of their capability to ferment dextrin has also received little confirmation[39]. Similarly, the diversity in the alcohol tolerance

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of different strains of yeasts does not show that this characteristic is 'peculiar to any species or genus'[40].

From these considerations the doubt naturally arises that the diversity in the fermentative ability of the related yeast types may be due to a difference in their chromosome constitution. The present paper gives the details of the experimental findings reported in an earlier communication[41].

MATERIALS AND METHODS

Strains of yeast. SUBRAMANIAM[42] demonstrated the diploid constitution of a brewery bottom yeast, BY 1, and by the treatment of the strain with acenaphthene for 90 days he isolated a strain, BY 3, having a tetraploid constitution[43]. It has been established that the tetraploid originated by a simple duplication of the chromosome complement of the control strain, BY 1. These two strains were employed throughout the present investigations. They have been kept under constant observation for the past five years. The diploid shows a seasonal variation in its giant colony characters whereas the tetraploid gives a smooth colony throughout the year[44].

Media. Two types of media were used. The synthetic salt-sugar medium contained sugar, KH_2PO_4 and $(NH_4)_2SO_4$. Commercial cane sugar was used in the fermentation experiments and pure glucose in other cases. The concentration of the sugar was varied as indicated in the experiments; the concentration of each of the two salts was maintained at 10% of the sugar present in the medium. The barley malt wort used had a sp.gr. of 1.020. This was prepared according to the schedule described by SUBRAMANIAM[45]. The pH was adjusted to 4.6–4.8 in all cases.

Inocula. The inocula were built up and adjusted in the following manner. One loop of the culture grown in barley malt agar for 24 hours at room temperature was inoculated into 5 ml of the wort. After growth for another 24 hours the contents of the tube were transferred under aseptic conditions into a litre Roux bottle containing 100 ml of wort. The bottle was kept in a flat position to enhance aerobic condition. The crop of yeast obtained after incubation for 24 hours at the room temperature was used as the inoculum after washing, resuspending in saline and making up the concentration to a known value under aseptic condition.

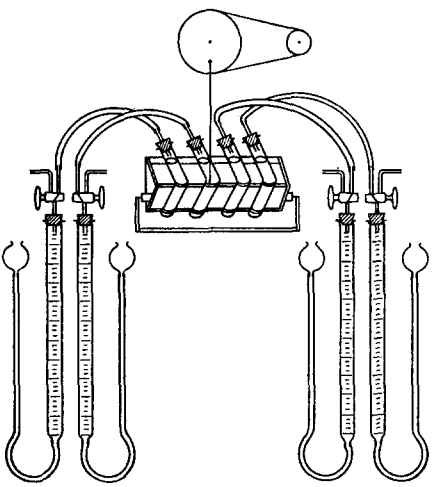

Fig. 1. Set of gasometers

Evaluation of the fermentative characteristic. Three independent criteria were employed for the comparison of the diploid and the tetraploid strains. i) The rate of attenuation of the salt-sugar medium. ii) the rate of evolution of carbon dioxide in fermentation of barley malt wort, and iii) sugar-alcohol balance in fermentation. In each case the results were verified by carrying out the experiments numerous times. Highly reproducible results were obtained and a representative set of observations recorded in each case. A set of gasometers was constructed for the purpose of the studies, and is diagramatically represented in Fig. 1. The fermentation tubes in the gasometer were mounted on an electrically driven rocker working at the rate of 80 to 90 strokes per minute. With the help of this equipment it is possible to study the relative rates of fermentation at room temperature for two strains at a time with duplicates for each. Periodic trial of quadruplicate fermentation in this apparatus showed that the maximum divergence of the reading of any individual gasometer does not exceed 2.5% from the mean recorded by the four.

EXPERIMENTAL RESULTS

1. *Attenuating power of the diploid and the tetraploid strains*

The medium was distributed in 100 ml conical flasks and was sterilised by autoclaving at 8 lbs steam pressure for 20 minutes. Proper aliquots of a concentrated medium were taken such that after the addition of the yeast suspension 25 ml of the final reaction mixture contained the desired percentage of sugar.

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The flasks were inoculated with aliquots of yeast suspensions prepared and standardised as above; the concentration of the yeast cells in the reaction mixture being identical for the two strains. Duplicate fermentations were run for each strain and incubations were at 28° C. The rate of attenuation in each case was followed up by noting the specific gravity of the reaction mixture at intervals with the help of a Westphal balance. The results of experiments with a 16% sugar medium is graphically represented in Fig. 2. The conc. of yeast cells was 24 mg per ml.

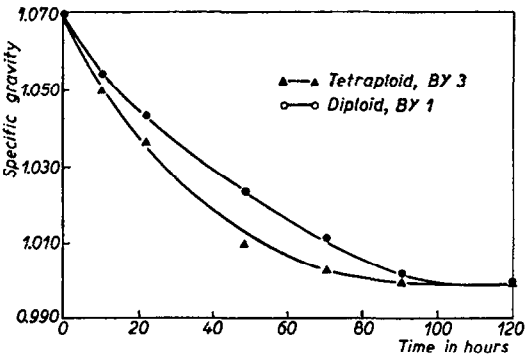

Fig. 2. Rate of attenuation of 16% sugar medium by the diploid and the tetraploid strains

It will be seen (Fig. 2) that the rate of fall in the specific gravity of the medium is much faster in the case of the tetraploid than in the diploid. By actual calculation of the loss in specific gravity, it is found that the total attenuation produced by the tetraploid at any time during the initial stages of fermentation is about 30 per cent. more than that given by the two-chromosome strain. This acceleration is, however, not discernible throughout the entire range of fermentation. The net result of this accelerated rate is reflected in the total time required by the two strains to reach the limiting attenuation. In the data presented, the fermentation is over in 90 hours in the case of the tetraploid whereas the diploid takes about 115 hours.

A very interesting phenomenon that is revealed by this experiment is the fact that although the tetraploid ferments much faster than the diploid, the final limit of attenuation reached by the two strains is identical (Fig. 2), which naturally means that the net amount of alcohol produced in both the cases is the same. The equalisation of the final attenuation suggested that the sugar concentration in the medium might be acting as the limiting factor for the continuance of fermentation by the tetraploid. Since strains have been reported[46] which are capable of producing as high as 15% alcohol in the wash, it was necessary to investigate the fermentation characters of the two strains with a higher concentration of the sugar in the medium. Accordingly, attenuation trials were conducted with media containing 24 and 32% sugar. The concentration of the inocula were 48 mg per ml and 36 mg per ml of the reaction mixture respectively in the two sets of experiments. The results are graphically represented in Figs 3 and 4 respectively.

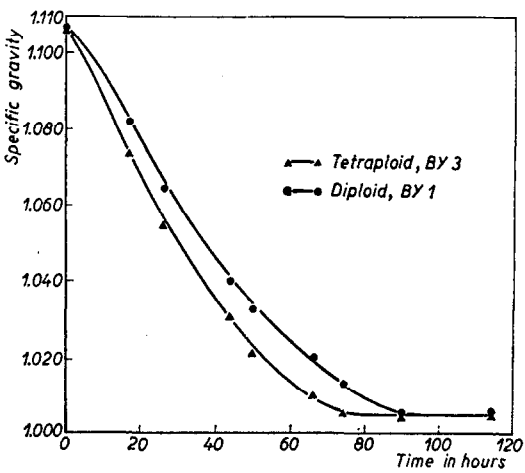

Fig. 3. Rate of attenuation of 24% sugar medium by the diploid and the tetraploid strains

On comparison of Figs. 2 and 3 it will be seen that no significant difference in the

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relative nature of the attenuation curves is noted on stepping up the initial concentration of sugar in the medium from 16 to 24%. Similar acceleration in the initial rate of fermentation in the case of the tetraploid and the final equalisation of the attenuation are equally discernible in either case. At 32% sugar concentration the results show certain anomalies. Although the relative difference in the rate of fermentation is still maintained (Fig. 4), the diploid is not able to reach the limit of attenuation produced by the tetraploid. Moreover, the rate of attenuation in the case of both the strains is extremely sluggish after about 90 hours of incubation. In order to have a better insight into the behaviour of the strains at high concentrations of initial sugar, the following experiment was carried out.

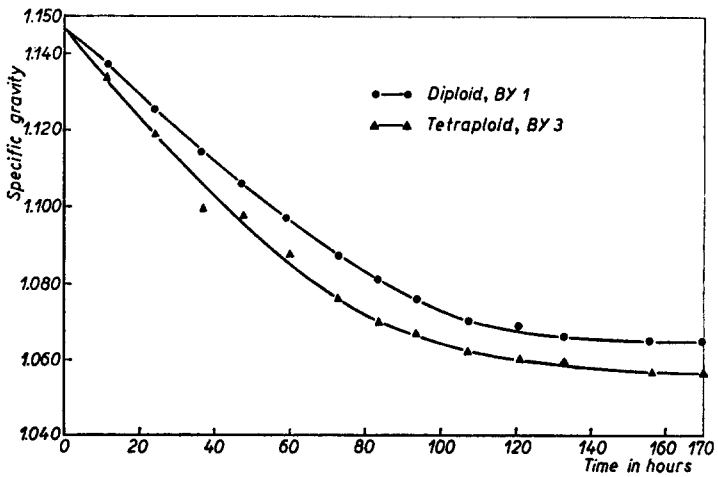

Fig. 4. Rate of attenuation of 32% sugar medium by the diploid and the tetraploid strains

2. Sugar tolerance of diploid and tetraploid strains

The stock medium employed for these studies contained 30% glucose and 3% each of the to salts.

Different aliquots of this medium were taken in bacteriological test tubes and the volumes made up to 10 ml in each case such that a series of media with gradually increasing sugar concentration were obtained. The media after sterilisation and cooling were inoculated with 1 ml of a saline suspension of yeast containing 50 mg of moist yeast cells. After 72 hours of incubation at 28° C fermentation was arrested by the addition of 0.5 ml of NaOH solution to each of the tubes and the amount of sugar left in each case estimated. Duplicate fermentations were run for each sugar concentration for either strain and sugar estimations were conducted by the method of STILES *et al.*[47] The mean of the sugar metabolised by the strains at different initial concentration of sugar have been graphically represented in mg per ml in Fig. 5A and as the percentage of initial sugar in Fig. 5B.

It will be seen from Fig. 5B that in the case of both the strains at lower concentrations of sugar almost 100% of the initial sugar is metabolised at the end of the specified period of incubation. Both the curves slope downward showing a marked fall in the percent metabolism with increasing concentration of sugar in the medium. According to the definition proposed by GRAY it will appear as though the tetraploid has a

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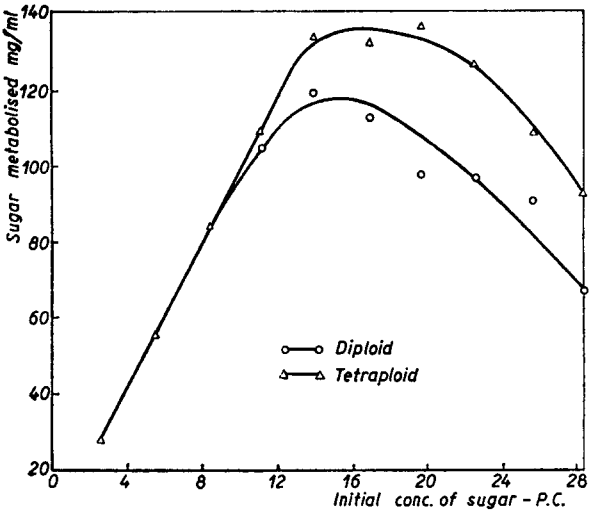

Fig. 5A. Sugar tolerance of the diploid and the tetraploid strains

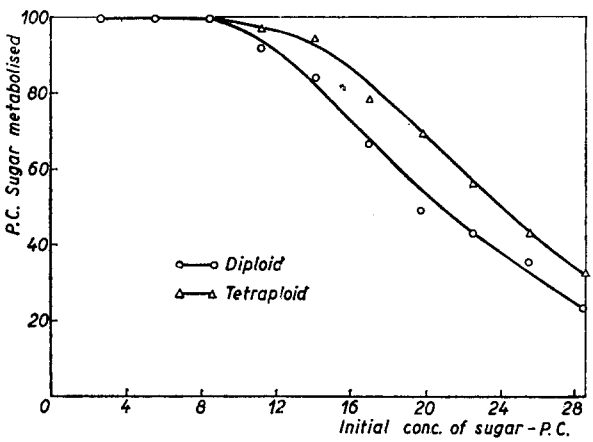

Fig. 5B. Sugar tolerance of the diploid and the tetraploid strains

slightly higher sugar tolerance since the curve for the strain "leaves the plateau" earlier. There are, however, certain difficulties in accepting this definition of sugar tolerance for the elucidation of the actual phenomenon. Strains having greater fermentative vigour will naturally show a higher percentage of sugar metabolism after 72 hours when the inoculum concentration and other experimental conditions are identical. The curve for such strains will show a later drop from the plateau irrespective of their actual sugar tolerance when compared with a low fermenting strain. In such cases, it may be suggested, the slope of the percent metabolism curve rather than its point of departure from the plateau would give a better idea of the relative sugar tolerances of the strains under comparison. The steeper the slope the greater is the susceptibility of the strain to the deleterious affect of increasing concentration of sugar. Judged on this basis there does not appear to be any difference in the behaviour of the diploid and the tetraploid strains in this respect since the curves for the two strains run almost parallel (Fig. 5B). On plotting the actual amount of sugar metabolised against the initial concentration of sugar from the same set of data it is seen (Fig. 5A) that there is a peak of sugar metabolism for each of the two strains and this is at about 18% sugar concentration for both.

These results indicate that the peculiar behaviour of the relative attenuation curves in the experiments with 32% sugar medium has apparently nothing to do with the sugar tolerance of the strain. The question of the duration of contact of the organism with the unfavourable environment, however, appears significant. This may contribute to the cessation of fermentation after a particular period of contact. During this period the tetraploid has already produced a higher degree of attenuation leading to the observed result (Fig. 3).

3. *Rate of evolution of caron dioxide by the diploid and the tetraploid strains*

The phenomenon revealed by the attenuation studies cannot be strictly designated

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as *fermentation* because there is considerable scope for the *growth* of yeast during such experiments. Through a study of the rate of evolution of carbon dioxide in the set of gasometer, it was hoped that a better idea of the actual fermentation characteristics under fairly anaerobic conditions could be obtained.

Preliminary trials were conducted in order to ascertain the amount of the inoculum necessary such that a complete series of observations could be made within 10 to 12 hours. On the basis of these trials 1 ml of yeast suspension containing 80 mg of yeast on the moist weight basis was fixed as the standard for both the strains, for the fermentation of 15 ml of barley malt wort. Duplicate fermentations were run for each strain for the comparative studies and the gasometers were read at different intervals. The results are given in table I. The amount of carbon dioxide evolved by the two strains during the successive hours have been indicated and the exact percentage differences in the amounts calculated.

TABLE I

RATE OF EVOLUTION OF CARBON DIOXIDE BY THE DIPLOID AND THE TETRAPLOID STRAINS

Time in hours	*Total volume of CO_2 recorded*		*CO_2 evolved in successive hours*		
	Diploid (ml)	*Tetraploid (ml)*	*Diploid (ml)*	*Tetraploid (ml)*	*Tetraploid / Diploid (%)*
0	—	—	—	—	—
0.5	2.3	3.0			
1.0	5.0	6.7	5.0	6.7	134.0
1.5	8.0	10.7			
2.0	11.4	15.3	6.4	8.6	134.3
2.5	15.5	20.7			
3.0	20.8	27.6	9.4	12.3	130.8
3.5	25.6	33.8			
4.0	31.5	41.6	10.7	14.0	130.8
4.5	38.2	50.8			
5.0	46.0	61.1	14.5	19.5	134.4
5.5	55.1	72.5			
6.0	64.8	83.9	18.8	22.8	121.3
6.5	76.4	94.3			
7.0	85.8	105.7	21.0	21.8	103.8
7.5	91.0	114.5			
8.0	100.2	122.4	14.4	16.7	116.0
8.5	107.9	129.8			
9.0	114.9	132.8	14.7	10.4	70.7
10.0	126.9	132.8	12.0	0.0	—
11.0	128.3	132.8	1.4	0.0	—

A perusal of the results would indicate that throughout the initial stages of fermentation the rate of evolution of carbon dioxide in the case of the tetraploid is about 30% faster than than that in the diploid. The total amount of carbon dioxide produced at the end of the fermentation is practically identical in both the cases. The results further show that the observed improvement in the rate of fermentation in the case of the tetraploid has apparently nothing to do with the powdery or flocculent nature of the strains, since the fermenting mixtures were kept in a homogeneous state by thorough agitation on the rocker.

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4. *Sugar-alcohol balance in fermentation by the diploid and the tetraploid strains*

These experiments were planned to elucidate the fermentative efficiencies of the strains. Fifteen ml aliquots of the salt-sugar medium were distributed in conical flasks of 50 ml capacity and the flasks were sterilised by autoclaving at 10 lbs of steam pressure for 30 minutes. The inocula for the two strains consisted of saline suspension of the cells prepared by the usual method and contained 0.15 mg of moist yeast cell per ml. Five ml of the suspension were used for each flask, duplicate having been run for each strain. The uninoculated flasks were used for the estimation of the initial concentration of the sugar in the medium.

After incubation for 48 hours at 30° C fermentation was arrested by the addition of NaOH solution. Yeast cells were removed by centrifugation and filtration for the estimation of the residual sugar and alcohol. All sugar estimations were conducted by the method of STILES *et al.*[47]; the method devised by JOHNSON[48] was employed for the estimation of alcohol. Efficiency data were obtained on the basis of one molecule of glucose producing two molecules of alcohol. The theoretical yield should thus be 0.511 g of alcohol per g of sugar utilised. Fermentative efficiency was obtained by dividing the actual yield by the theoretical yield, both calculated on the basis of fermentation of 100 g of sugar. The results are presented in Table II.

TABLE II

SUGAR-ALCOHOL BALANCE IN FERMENTATION BY THE DIPLOID AND THE TETRAPLOID STRAINS

Strain	*Sugar (mg/ml)*		*Alcohol (mg/ml)*		*Fermentative efficiency* (%)
	Initial	*Utilised*	*Estimated*	*Theoretical*	
Diploid	172.50	114.20	56.96	58.35	97.61
Tetraploid	172.50	147.02	74.76	75.13	99.51

It will be seen from the table (Table II) that at the end of 48 hours of fermentation the amount of alcohol produced by the tretaploid is about 30% more than that given by the diploid strain. The data presented further reveal that the tetraploid has got a slightly better efficiency, which only shows that under the conditions employed for the experiments more of the sugar was utilised for cellular synthetic processes in the case of the diploid than in the case of the tetraploid. This contention is supported by our observation[50] on the yield of the yeast crops produced by the two strains under various conditions of aerobiosis.

DISCUSSION

Precise data are not available on the *rates* of fermentation of the related yeast types so often quoted by different workers. In most cases the emphasis has been placed on the *degree* of attenuation rather than the *rate*. A consideration of the definition of fermenting power[34] indicates that under favourable conditions comparable strains should produce the same degree of final attenuation provided that the theoretical maximum of alcohol produced does not act as the limiting factor. The rates of attenuation produced by them may, however, vary widely. Under ideal conditions of fermentation the tetraploid maintains a superiority in the rate of fermentation practically throughout the entire period,

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although the final degree of attenuation produced by it is the same as given by the diploid.

Fresh moist weight of yeast has been taken as the criterion for comparison since it is known that one of the primary effects of polyploidy is reflected in the moisture relationship of the organism[49]. It would be realised that the functional unit is the living yeast cell and not the dry matter it contains. Consequently the improved functioning of the tetraploid is demonstrable even if the comparisons are made on the dry weight basis. The improvement in the fermentative vigour on duplication of the chromosome complement finds a rational explanation[51] on the basis of endopolyploidy in yeasts observed by SUBRAMANIAM[52].

Divergence in fermentative ability among the genetically variant strains of yeasts is not altogether a rare finding although an induction of polyploidy as the cause has never been put forward. The peculiar fermentative behaviour of the "reduced forms" of yeasts obtained by SATAVA[53] and of the "S" and "R" forms of yeasts reported by FABIAN AND WICKERHAM[54] may find a rational explanation on the basis of the present studies.

In considering the industrial implications of these findings it should clearly be borne in mind that such a method of improvement cannot be applied to each and every strain. There is good reason to believe that most of the strains now employed in industrial practice are polyploids chosen by natural selection and consequently the attempts at further duplication of the chromosome complement will, in all probability, be futile. However, since the direct improvement of the polyploid strains by hybridisation[55,56] is also a virtual impossibility, it is always worthwhile to recover the haploid strains for such work. Once the superior genic combination is achieved by hybridisation, a further accentuation of the desirable traits can be accomplished through an induction of polyploidy.

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SUMMARY

Yeast strains belonging apparently to the same species vary widely in their fermentation characteristics and the cause of these variations has not been established on any scientific basis. There is the possibility that this difference is purely governed by the chromosomal constitution of the different strains. Direct and indirect evidences are presented in support of this contention. A diploid strain of brewery bottom yeast and an autotetraploid obtained from it by treatment with acenaphthene were used as the experimental materials.

The data presented show that under a variety of conditions the tetraploid strain shows a faster rate of fermentation—the acceleration being about 30% over that given by the diploid. Data on sugar-alcohol balance indicate that the tetraploid has got a slightly better fermentative efficiency. Although the rate of fermentation varies widely, the final amount of alcohol produced by the two strains is identical. There has been no change in the sugar tolerance on a duplication of the chromosome complement. The induction of polyploidy in conjunction with hybridisation could be used as a profitable method for the improvement of yeast strains for industrial practice.

RÉSUMÉ

Des souches de levure faisant partie, apparement, de la même espèce, varient largement dans

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leurs caractéristiques de fermentation; la cause de ces variations n'a pas été établie sur des bases scientifiques. Il se peut que cette différence soit gouvernée simplement par la constitution chromosomique des différentes souches. Nous présentons des arguments directs et indirects en faveur de cette hypothèse. Nous avons employé pour nos expériences une souche de levure de bière à fermentation basse diplo de et une souche autotetraplo de obtenue par traitement de la première à l'acénaphthène.

Il ressort des données présentées que, dans des conditions diverses, la souche tetraplo de montre une vitesse de fermentation d'environ 30% plus élevée. Des données concernant le bilan sucre-alcool montrent que la souche tetraplo de donne un rendement un peu meilleur. Bien que la vitesse de fermentation varie dans de larges mesures, les quantités d'alcool produites par les deux souches sont indentiques. Il n'y avait pas de changement de la tolérance de sucre à la suite de la duplication du nombre de chromosomes.

L'induction de la polyplo die, conjointement avec l'hybridation, pourrait être employée utilement pour améliorer les souches de levure employées dans la pratique industrielle.

ZUSAMMENFASSUNG

Hefestämme, welche offensichtlich derselben Art angehören, variieren bedeutend in ihren Gärungseigenschaften. Die Ursache dieser Variationen wurde wissenschaftlich nicht festgestellt. Es besteht die Möglichkeit, dass sie einfach durch die Chromosomen-Zusammensetzung der verschiedenen Stämme beherrscht werden. Direkte und indirekte Beweise für diese Hypothese werden angeführt. Ein diploider Stamm von untergäriger Bierhefe und ein autotetraploider Stamm, welcher aus ersterem durch Behandlung mit Acenaphthen erhalten worden war, dienten uns als Untersuchungsmaterial.

Die angeführten Zahlen zeigen, dass unter verschiedenen Bedingungen der tetraploide Stamm eine etwa 30% höhere Gärungsgeschwindigkeit zeigt als der diploide. Aus Angaben über Zucker-Alkohol-Bilanzen kann man schliessen, dass der tetraploide Stamm eine etwas höhere Ausbeute gibt als der diploide Stamm. Obwohl die Gärungsgeschwindigkeiten sehr verschieden sind, liefern doch beide Stämme die gleiche Menge Alkohol. Die Zuckertoleranz wurde durch die Verdopplung des Chromosomensatzes nicht beeinflusst. Die Induktion der Polyploidie könnte, zusammen mit der Kreuzung, als nützliche Methode zur Verbesserung der in der Industrie verwendeten Hefestämme dienen.

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