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MANUEL LOSADA

THE HYDROLYSIS OF RAFFINOSE BY YEAST MELIBIASE AND THE FERMENTATION OF RAFFINOSE BY COMPLEMENTARY GENE ACTION

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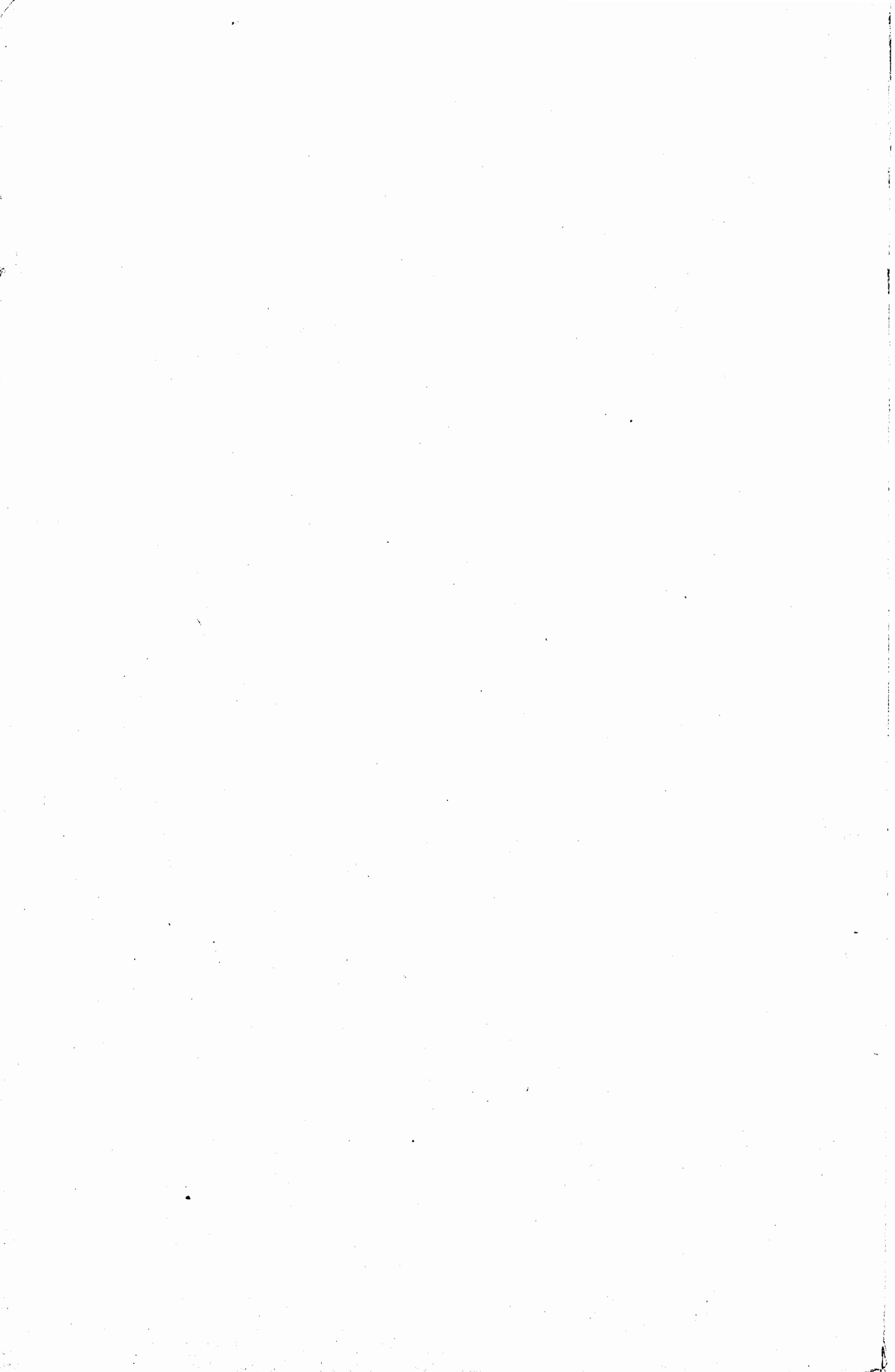

THE HYDROLYSIS OF RAFFINOSE BY YEAST MELIBIASE AND THE FERMENTATION OF RAFFINOSE BY COMPLEMENTARY GENE ACTION

BY

MANUEL LOSADA[1]

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INTRODUCTION

Many of the complications which arise in the study of carbohydrase specificity involve the difficult task of isolating the enzymes in a pure state, and it is often not possible to ascertain whether one is working with a single enzyme or a mixture of several.

In this connection, special significance can be attached to the carbohydrases in yeasts, since in these organisms it is possible through genetic methods to isolate single spore cultures containing either one or several genes for the synthesis of these enzymes. It is thus possible to investigate particular carbohydrases without the interfering effects of others by employing a culture known to contain only the genes

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responsible for their production. As examples can be mentioned the following genes isolated by WINGE and ROBERTS (1952, 1956): 1) The *Me*-gene, responsible for the production of melibiase (= α-galactosidase); 2) The *M*-genes, responsible for the production of α-glucosidase (= maltase), of which M_1 and M_4 (glucosaccharase = glucoinvertase) hydrolyze both maltose and sucrose and M_2 (maltase, *sensu strictu*) hydrolyzes maltose alone; and 3) The *R*-genes, responsible for the production of β-h-fructosidase (= fructoinvertase), which hydrolyzes raffinose and sucrose. Furthermore, through the use of suitable hybridizations and subsequent segregations, it is possible to obtain genotypes with any desired combination of genes, and it is at once evident that such genotypes can be of the greatest interest in connection with the fermentation of sugars composed of different monosaccharides with various types of glycosidic linkages. For example, the trisaccharide raffinose, which we have investigated in the present paper, contains three different bonds: α-galactosidic, α-glucosidic, and β-h-fructosidic.

WEIDENHAGEN (1940) postulated that only five types of glycosidases exist (α- and β-glucosidases, α- and β-galactosidases, and β-h-fructosidase). His experiments led him to the conclusion that the carbohydrases possess only one affinity for the glycosidic portion of the substrate molecule. Therefore the same type of enzyme, α-D-glucosidase, splits all α-D-glucosidic linkages in naturally occurring oligosaccharides (maltose, sucrose, turanose, melezitose and trehalose) and in synthetic α-D-glucosides (heterosides), and the same type of enzyme, α-D-galactosidase, splits the α-D-galactosidic bond in melibiose and raffinose. But Weidenhagen's theory lacks that universal validity which permits an *a priori* conclusion as to whether a particular enzyme hydrolyzes a particular glucosidic bond or not. Some of the well-known objections to this theory include the following:

1) Some carbohydrases hydrolyze maltose but not α-methyl-glucoside, in spite of the fact that both substances contain a terminal α-D-glucopyranoside ring.
2) Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), in spite of the fact that it is a α-D-glucoside, is not hydrolyzed by yeast maltase. This hydrolysis requires the presence of a specific enzyme, trehalase.
3) α-glucosidases are known which hydrolyze maltose and sucrose, and others which hydrolyze maltose but not sucrose.

NEUBERG's investigations on the splitting of raffinose (1907) into galactose and sucrose by the melibiase (α-galactosidase) from emulsin do not permit the conclusion that the melibiase occurring in yeasts is also capable of splitting raffinose. Probably the yeast melibiase has been heretofore inadequate for demonstrating raffinose hydrolysis because of the simultaneous presence of β-h-fructosidase in the yeast cells employed. This latter enzyme removes the fructose portion of the raffinose molecule, liberating melibiose. However, now it is possible through genetic methods to isolate single spore cultures containing only the gene *Me* responsible for the production of melibiase, and therefore the raffinose hydrolysis brought about by this enzyme can be investigated without the interfering effect of the β-fructosidase.

On the basis of his experimental work, LINDEGREN in "The Yeast Cell" is led to the following conclusions:

1) "Melibiase is not capable of hydrolyzing melibiose in the raffinose molecule".
2) "The complete hydrolysis of raffinose depends on the presence of both melibiase and sucrase, the latter acting first".

It is my opinion, as will be discussed later, that the experimental work of LINDEGREN does not support these two conclusions.

WINGE and ROBERTS (1956) demonstrated that raffinose may be fermented by yeast cultures of the genotype *Me G r* (the *Me*-gene producing melibiase and the *G*-gene producing galactozymase), which lack the *R*-gene. As pointed out by these authors, this observation can only be explained by the occurrence of complementary action between *Me* and *G*, assuming that melibiase hydrolyzes the raffinose, liberating galactose—in other words, yeast melibiase is capable of splitting raffinose, just as it had previously been shown for emulsin melibiase.

The present work, undertaken by methods to be presently described, offers proof of the assumption that raffinose can be hydrolyzed by the action of yeast melibiase. As a result of this work and according to Weidenhagen's theory, it appears that it is preferable to designate this enzyme α-D-galactosidase rather than melibiase or galactoraffinase. The methods which have been employed in order to confirm the existence of this hydrolysis have led to new and interesting examples of complementary gene action.

CHEMICAL AND GENETICAL BASIS FOR THE METHODS EMPLOYED IN DEMONSTRATING RAFFINOSE HYDROLYSIS

The structure of raffinose may be written as follows: 6-α-D-Galactopyranosyl ⟨ α-D-glucopyranosyl ⟨ ⟩ β-D-fructofuranoside, and thus none of its 3 reducing groups from the 3 monosaccharides are free. It is for this reason that raffinose itself neither reduces Fehling's solution nor yields osazone from phenylhydrazin. The hydrolysis of raffinose by the action of melibiase can be demonstrated with Fehling's solution. That the hydrolysis has led to the formation of galactose and sucrose can be demonstrated by the aid of phenylhydrazin. (Only the galactose produces osazone, since the sucrose has no free reducing group). The galactosazone produced can be identified microscopically as well as by melting point determinations.

The galactose and sucrose which should be formed as the result of the hydrolysis of the raffinose molecule by a *Me*-culture cannot be directly fermented by the zymase complex of the cell, but their presence can be demonstrated by employing cultures of other genotypes which bring about their fermentation: galactose is fermented by a *G*-culture, and sucrose is fermented by an *R*-culture or by certain *M*-cultures (M_1 and M_4). Cultures containing the *R*-genes are of course unsuitable for these investigations on raffinose hydrolysis, since the β-h-fructosidase synthesized by these *R*-genes not only hydrolyzes sucrose into fructose and glucose but also raffinose into fructose and melibiose. Since fructose and glucose are fermented by all yeasts capable of fermentation, the use of a culture containing the *R*-gene will always give a positive fermentation result, regardless of whether raffinose had or had not been previously hydrolyzed. On the other hand, cultures containing either M_1 or M_4 and which synthesize α-glucosidases are most useful in such investigations, for they do not act upon raffinose itself, in which the glucose portion of the molecule is protected by being bound to the galactose portion, but they hydrolyze and therefore ferment the sucrose which is liberated during the removal of the galactose portion from the raffinose molecule by the action of melibiase. These enzymes act therefore as α-glucosidases (glucoinvertases) and not as β-h-fructosidases (fructoinvertases), and raffinose will be only fermented, if it has been previously hydrolyzed.

LINDEGREN maintains, quite rightly, that *Me su*-cultures (*Su* and *R* both synthesize β-h-fructosidase) are unable to ferment raffinose, but this leads him to the erroneous conclusion that melibiase

is unable to hydrolyze raffinose. When one takes into consideration that the two sugars, galactose and sucrose, which are produced through the hydrolysis of raffinose by melibiase cannot be directly fermented by the zymase present in all fermenting yeast cells, it is not difficult to understand why *Me su*-cultures are unable to ferment raffinose, even though hydrolysis has taken place. This lack of fermentation does not permit the conclusion that hydrolysis is also lacking.

In addition to the qualitative measurements of fermentation just discussed, it is also possible to undertake quantitative measurements on cultures of different genotypes, in which different degrees of raffinose fermentation (1/3, 2/3, 3/3) can be demonstrated, depending upon the number of monosaccharides which are fermented, following their liberation at hydrolysis.

Cultures which originate from single spores containing one of the following three genes, *Me*, *G*, or *M*, are unable to ferment raffinose. On the other hand, if the melibiase is able to hydrolyze raffinose, cultures of the genotype *Me G* or *Me M* will ferment raffinose. The complementary action of *Me* + *G* has been investigated in much detail by WINGE and ROBERTS (see the accompanying publication), while the action of *Me* + *M* will be discussed in this paper.

TECHNIQUES EMPLOYED AND RESULTS OBTAINED

All the yeasts employed in this study were diploid cultures of known genotype from the collection at the Carlsberg Laboratorium.

1. Chemical Methods.

Before commencing these investigations, I was aware of the possibility that it might not be possible to investigate raffinose hydrolysis by employing living cultures containing the *Me*-gene. The results obtained have, however, shown that living *Me*-cells do in fact hydrolyze raffinose, permitting the demonstration by the methods discussed below of the presence of liberated galactose and sucrose. It has consequently not been necessary to work with enzymatic extracts which would have complicated the subsequent fermentation experiments (described in the following section) which naturally require the use of sterile solutions, but without the addition of toxic chemical substances. We have, of course, employed sterile technique throughout the course of these investigations.

In order to demonstrate the presence of raffinose hydrolysis with the aid of Fehling's solution we have employed the following method:

a) The Cultures: Small glass test tubes containing 2–3 cc. of 2 % glucose yeast water are inoculated with young cultures of the genotypes *Me* (303-49, 303-53, 306-43), R_2 (303-67), and as a control, *me* r_2 (303-69). After 2 days' incubation at 25° C. the cultures are decanted and the cells are washed in sterile tap water. After centrifugation, the cultures are again decanted, and since the supernatant gives no reaction with Fehling's solution, it is unnecessary to rewash the cells.

b) The Hydrolysis: These 3 cultures are tested for their ability to hydrolyze raffinose and sucrose. To each culture is added 2 cc. of either 4 % raffinose yeast water or 2 % sucrose yeast water. The cultures are then incubated from 1–2 days at 25° C.

c) The Fehling Test: After incubation the cultures are decanted and whether hydrolysis of the sugars in question has occurred is determined by the use of Fehling's solution (which, as is well-known, yields a positive reaction (i. e., is reduced) in the presence of glucose, fructose, galactose, and melibiose, and a negative reaction in the presence of sucrose and raffinose). The results obtained are shown in Table 1.

Table 1.

Results obtained by employing the Fehling reaction to determine the ability of different genotypes to hydrolyze sucrose and raffinose.

Culture		Sucrose Hydrolysis	Raffinose Hydrolysis
Number	Genotype		
303-49 303-53 306-43	*Me* r_2	—	+
303-67	*me* R_2	+	+
303-69	*me* r_2	—	—

Since melibiase, as expected, was unable to hydrolyze sucrose, it is evident that the hydrolysis of raffinose by this enzyme involved the melibiose portion of the molecule rather than the sucrose portion. The *me* r_2-genotypes gave negative Fehling's reaction with respect to both sucrose and raffinose.

After the course of 24 hours the *Me* r_2 genotype gave a clearly positive reaction for raffinose, and the *me* R_2 genotype gave an even

stronger positive reaction. This difference is explainable on the assumption that melibiase hydrolyzes raffinose more slowly than β-h-fructosidase. Experiments which will be described later on seem to confirm this assumption, but it must be taken into consideration, together with other possibilities, that the β-h-fructosidase synthesized by R_2 liberates two reducible sugars (fructose and melibiose), while the melibiase synthesized by *Me* liberates only one (galactose).

The technique employed in the identification of the liberated galactose, was as follows:

a) The Cultures: 303-49 and 303-53, both of which were of the genotype $Me\,r_2$, and 303-69 ($me\,r_2$) were employed after the same treatment as described under 1a, page 465.

b) The Hydrolysis: These 3 cultures were tested for their ability to hydrolyze raffinose. The technique was essentially similar to that described under 1b except that incubation took place at 32° C and lasted 3 days.

c) The Phenylhydrazin Reaction: After centrifugation the cultures were decanted, and 5 cc. of the supernatant from each of the 3 cultures was poured into a test tube, to which was added 1 g. sodium acetate. After boiling for 5 minutes in a water bath, the test tubes were cooled, the contents filtered, and to the filtrate was added 1 cc. of a fresh 10 $^0/_0$ solution of phenylhydrazinchloride, after which the solutions were boiled for $1^1/_2$ hours in a water bath.

Raffinose which had been in contact with the *Me*-cultures produced yellow osazone crystals, while raffinose which had been treated with the *me*-culture gave a bright yellow color but practically no precipitation. This weak positive reaction can be explained by the hydrolysis of raffinose due to the prolonged boiling ($1^1/_2$ hours).

The identification of the osazone which had been formed was carried out by determination of its melting point in a glycerin bath. The crystals melted with a dark color at 180.5° C. (corrected temperature). The heating was very gradual, at the rate of ca. 0.5° C. pr. minute. The osazone must therefore be considered to be galactosazone.

2. Qualitative Fermentation Tests.

To demonstrate the presence of the galactose which is liberated at raffinose hydrolysis by melibiase, fermentation tests are carried out with yeast cultures containing the gene for galactozymase syn-

thesis, G. The presence of sucrose is likewise investigated by employing cultures containing M_1 or M_4, while M_2-cultures are used as controls, since the α-glucosidase they produce is able to hydrolyze maltose but not sucrose. In view of the fact that all the yeast cultures of the Carlsberg Collection which have been analyzed genetically with respect to galactose fermentation and which have been found to lack the G-gene contain g_s (slow synthesis of galactozymase), it is to be expected that raffinose which has already been hydrolyzed by melibiase will be fermented by M_2-cultures. It is obvious that the fermentation process will be slower with M_2-cultures than with G-, M_1-, or M_4-cultures.

Raffinose is hydrolyzed by 2 day old cultures containing Me (303-49, 303-53) during the course of 3 days at 32° C. As a control, me-cultures of the same age (303-69) are employed (see Table 1) After the raffinose is hydrolyzed, the solution is centrifuged and fermentation of the sucrose and galactose formed as a result of this hydrolysis is undertaken in the Winge fermentometer at 25° C. by using cultures containing G (303-17, 303-43), M_1g_s (177-248), M_4g_s (193-2), and M_2g_s (186-17).

Table 2 shows the results obtained by these experiments. The raffinose which was hydrolyzed by the Me-cultures was rather rapidly fermented (1–4 days) by G-, M_1-, or M_4-cultures and very slowly fermented (15–20 days) by M_2g_s-cultures. In these slow fermentations it was not sucrose, but galactose, that was fermented through the activity of the g_s-gene. The raffinose which was treated with the me-culture and which consequently was not hydrolyzed was not fermented in any case.

Table 2.

The effect of various genotypes on the fermentation of hydrolyzed and non-hydrolyzed raffinose.

Culture		Fermentation of Raffinose previously treated with cultures:	
Number	Genotype	303-49 (Me) and 303-53 (Me)	303-69 (me)
303-17, 303-43	G	+ (R)	—
177-248	M_1g_s	+ (R)	—
193-2	M_4g_s	+ (R)	—
186-17	M_2g_s	+ (S)	—

R = rapid S = very slow

3. Quantitative Fermentation Tests.

According to the foregoing, it may be concluded that through the activity of yeasts of different genotypes the raffinose molecule may be

1) partially hydrolyzed by the activity of the *R*-gene into fructose and melibiose and by the *Me*-gene into galactose and sucrose, or
2) completely hydrolyzed by the combination of *Me* + *M* or *Me* + *R* into fructose, glucose, and galactose.

The liberated fructose and glucose are always fermented by all yeasts containing the zymase complex, while galactose is fermented only by those yeasts containing *G*. The degree of raffinose fermentation obtained therefore depends upon the genotype of the culture employed and may be expressed as 0, 1/3, 2/3, or 3/3.

In what follows we shall study the hydrolytic and fermentative ability of some of the genotypes listed in Table 3 with regard to raffinose.

Table 3.

Hydrolysis and fermentation of raffinose by *Saccharomyces* cultures of different genotype.

Genotype	Hydrolytic Activity	Sugars produced by the hydrolysis	Fermented fraction of the raffinose molecule
M	—	0	0
Me	+	Galactose Sucrose	0
R	+	Fructose* Melibiose	1/3
Me G	+	Sucrose Galactose*	1/3
Me M	+	Fructose* Glucose* Galactose	2/3
Me R	+	Fructose* Glucose* Galactose	2/3
Me M G	+	Fructose* Glucose* Galactose*	3/3
Me R G	+	Fructose* Glucose* Galactose*	3/3

* Monosaccharides which are fermented

For quantitative analysis of fermentative ability, the van Iterson Kluyver fermentometer was employed in all cases. The following cultures were employed in these tests: 307-70 ($Me\ M_1\ M_6\ G$), A-10 ($Me\ M_1\ g_s$), C-1 ($Me\ M_4\ g_s$), A-34 ($Me\ g_s$) and C-2 ($M_4\ g_s$). The cultures were prepared as follows: 2 Freudenreich flasks containing 8 % Pilsner wort were inoculated with the culture and incubated for 2 days at 25° C; the cultures were then decanted, and the cells were suspended in sterile tap water, centrifuged, and again decanted. These cells were then suspended in 1.5 cc. 4 % raffinose yeast water or 2 % sucrose yeast water which were inoculated into the fermentometer, but only 1 cc. was used to measure fermentation. The fermentometers were kept at 25° C. during the course of the experiment. The barometric pressure was normal. Theoretically, 1 cc. of a 4 % solution of raffinose (raffinose, cryst. 5 H_2O) under these conditions should yield 3.3, 6.6, or 9.9 cc. CO_2 depending upon whether the degree of fermentation is 1/3, 2/3, or 3/3. One cc. of a 2 % solution of sucrose under the same conditions should theoretically yield 5.7 cc. CO_2. Small deviations are to be expected in these fermentometers. The first cubic centimeter in the diagram corresponds more or less to the volume of the solution employed.

The fermentative ability of five different genotypes, *ME*, *M*, *ME G*, *ME M*, and *ME M G*, will now be considered in light of the results obtained and the results theoretically expected.

I. *Me*. (See Fig. 2, Culture A-34). Synthesizes α-D-galactosidase (= melibiase) and therefore is able to split raffinose into galactose and sucrose, which it cannot ferment. Hydrolysis partial, degree of fermentation 0. Since a *Me*-culture possesses the g_s-gene, it is expected that it can bring about a very slow fermentation, degree 1/3. After the course of a few days, fermentation commences but proceeds very slowly, as can be seen from the curve.

II. *M*. Synthesizes α-glucosidase and is therefore able to split maltose and sucrose (M_1, M_4) or only maltose (M_2). The M_2-cultures are naturally unable to hydrolyze raffinose, but neither can the M_1- or M_4-cultures, since the glucose portion is protected by the galactose portion of the molecule. Hydrolysis negative, degree of fermentation 0.

With regard to raffinose hydrolysis by the α-glucosidases, synthesized by the *M*-genes, some peculiar results were obtained which are worthy of mention. During an investigation of the Fehling's

reaction of raffinose solutions which had been treated with M_1-, M_2-, and M_4-cultures, it was found, contrary to expectation, that the raffinose from the M_1-cultures gave a positive reaction (after 1 day weak, but after several days more pronounced), while the raffinose from the M_2- and M_4-cultures gave a constant negative reaction. It was, however, not possible to establish with certainty whether the α-glucosidase synthesized by M_1 is able to hydrolyze the raffinose molecule, for repeated experiments with several different M_1-cultures gave sometimes positive (after 1 or several days), sometimes completely negative reactions (even after 15 days). The inability of M_1-cultures to ferment raffinose seems to indicate that this α-glucosidase is unable to hydrolyze raffinose, although it is possible that if hydrolysis can take place, it occurs to only such a small extent that fermentation is not observable. It was also contrary to expectation that sucrose solutions which had been treated with M_1- and M_4-cultures (both of which ferment maltose and sucrose) gave a negative reaction with Fehling's even after the course of several days. This result was even more strange in view of the fact that the sucrose which had been treated with R_2-cultures gave a very strong reaction in the course of 1 day. Perhaps the hydrolysis brought about by α-glucosidase only takes place within the cell, while the β-h-fructosidase may be localized in the cellular membrane, bringing about extracellular hydrolysis.

III. *Me G.* (See WINGE and ROBERTS, accompanying paper). Hydrolysis partial (galactose and sucrose), degree of fermentation 1/3 (galactose).

IV. *Me M.* (See Fig. 2, Cultures A-10 and C-1). The raffinose molecule is split by the α-galactosidase synthesized by the *Me*-gene, and this hydrolysis permits the liberated sucrose to be split by the α-glucosidase synthesized by either M_1 or M_4. Of the three monosaccharides which are liberated, only the glucose and the fructose are fermented. Hydrolysis complete, degree of fermentation 2/3. Also in this case, the presence of g_s causes a very slow fermentation of the galactose so that the final degree of fermentation should be 3/3.

Here, then, we are dealing with an hydrolysis in two phases which eventually results in the formation of the 3 monosaccharides of which the raffinose molecule is composed.

In order that the α-glucosidase can become active, it is essential that the α-galactosidase has already liberated the galactose portion

of the raffinose molecule. Thus, when both M_1 or M_4 and Me are present together in one culture a total hydrolysis will occur, with resultant 2/3 fermentation (see Fig. 1).

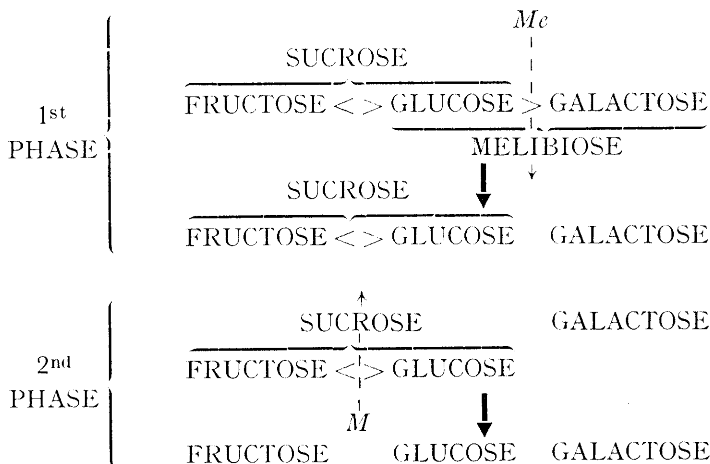

Fig. 1. Scheme of the complete hydrolysis of the raffinose molecule through the complementary action of *Me* (α-D-galactosidase) and *M* (α-D-glucosidase).

An important distinction exists between the hydrolytic activity of $Me + M$ and that of $Me + R$. In the latter case, although the hydrolysis is also complete and the degree of fermentation is 2/3, it cannot be established with certainty whether the melibiase is capable of hydrolyzing the raffinose, since we know that β-h-fructosidase is able to hydrolyze raffinose, making the melibiose available to the attack of melibiase.

The study of raffinose fermentation by *Me M*-cultures shows, contrary to Lindegren's claim, that even in the absence of β-h-fructosidase, complementary action can bring about total hydrolysis of the raffinose molecule. The hydrolysis of the melibiose portion of the molecule may occur, therefore, even if the β-h-fructosidase has not first removed the fructose portion of the molecule. LINDEGREN maintains that melibiase is not capable of hydrolyzing melibiose in the raffinose molecule and that the total hydrolysis of raffinose takes place when the β-h-fructosidase, acting first, permits the activity of melibiase. In the total hydrolysis brought about by complementary action ($Me + M$) the sequence of events is reversed—i. e., melibiase hydrolysis takes place first, followed by α-glucosidase hydrolysis.

V. *Me M G.* (See Fig. 2, Culture 307-70). Here complete hydro-

lysis and complete fermentation (3/3) is attained. The zymase complex of the yeast ferments the glucose and fructose directly, and the galactose is fermented by the aid of the enzyme which is synthesized by the *G*-gene.

From the accompanying paper of WINGE and ROBERTS as well as from the present paper it appears that raffinose is more rapidly fermented by R_2-cultures (direct fermentation following hydrolysis through the activity of β-h-fructosidase) than by *Me G*- or *Me M*-cul-

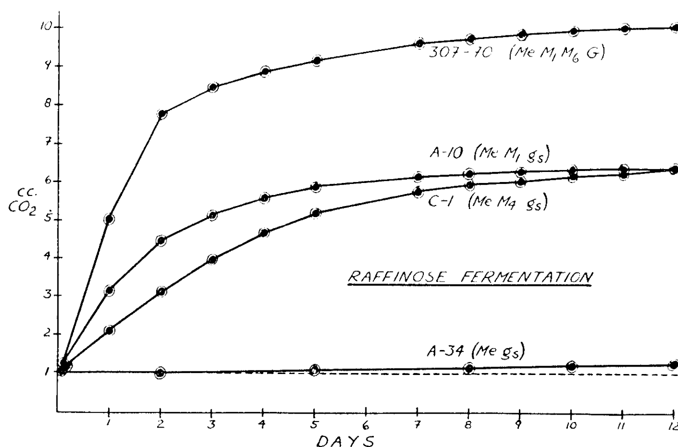

Fig. 2.

tures [fermentation following complementary action in 2 phases: 1) hydrolysis by α-galactosidase and 2) transformation of galactose by galactozymase (*Me G*) or hydrolysis of the liberated sucrose by α-glucosidase (*Me M*)]. The slowness with which raffinose is fermented by these two processes of complementary action may thus have two causes: 1) slow hydrolysis of raffinose by melibiase and 2) slow galactose transformation ($Me + G$) or slow sucrose hydrolysis by α-glucosidase ($Me + M$). A consideration of the curves seems to demonstrate that the first cause may be most important. A comparison of the curve C-2 (Fig. 3) obtained from 2 $^0/_0$ sucrose and the curve C-1 (Fig. 2) obtained from 4 $^0/_0$ raffinose shows that most of the original sucrose is fermented within 8–10 hours by α-glucosidase (M_4), while it takes five days for the sucrose which is liberated by the melibiase-hydrolysis of raffinose to be fermented.

The curve *Me M G* and WINGE and ROBERTS' curves of R_2, $Me\ R_2$, and $Me\ R_2\ G$ show that here at least the galactose is

not more slowly fermented than the two other monosaccharides, fructose and glucose, which are liberated at raffinose hydrolysis.

It therefore appears that both sucrose hydrolysis by α-glucosidase as well as galactose fermentation may take place rapidly. The slowness with which raffinose is fermented by complementary action seems therefore to be due to the hydrolysis of raffinose by melibiase rather than the hydrolysis of sucrose by α-glucosidase or the transformation of galactose by galactozymase.

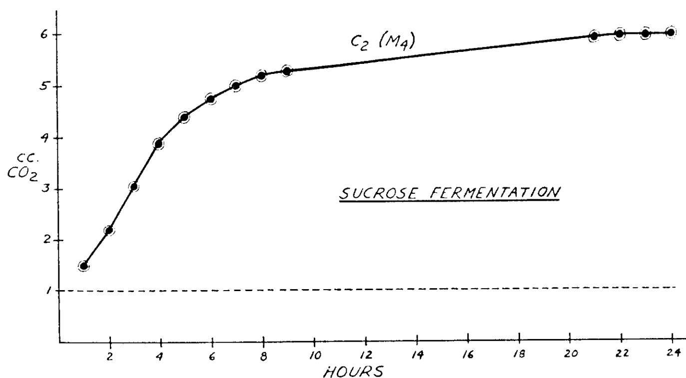

Fig. 3.

Winge and Roberts' curves for MeR_2 and $Me\,G\,R_2$ appear at first glance to contradict this view, but, actually, although the melibiase in these cases takes part in the hydrolysis of raffinose, it is possible that it does not attack the raffinose molecule itself but the melibiose that is liberated by the hydrolytic action of β-h-fructosidase. A consideration of the $Me\,M$-curves in which melibiase must necessarily act upon raffinose, also appears to confirm this assumption.

Apart from other possible factors, the shape of both $Me\,M$-curves may be due not only to the fact that the first phase of the hydrolytic process is slow (here the repressing action of the galactose which is liberated may have some effect), but also that the second phase, the splitting of sucrose, may be delayed by a competitive action between the liberated sucrose and the sucrose portion of the intact raffinose for the possession of the M-enzymes. (The experiment described on page 479 seems, however, to indicate that there is no competition between raffinose and sucrose).

4. The *Me* × *M* Hybrids.

In the production of these hybrids the methods employed were those of WINGE & LAUSTSEN (1938), and in all cases spore × spore crossings were undertaken. In the micromanipulation chambers, Pilsner wort droplets were employed, and for sporulation, Fowell's medium was used. The hybrids and their single spore progeny were maintained in 8 % Pilsner wort.

Two different hybridizations were attempted:

1) $Me \times M_1$
2) $Me \times M_4$

The parents employed in these hybridizations were the following diploid single spore cultures:

306-43 ($MeMe\ rr\ m_1m_1\ g_sg_s$)
177-248 ($meme\ rr\ M_1M_1\ g_sg_s$)
193-2 ($meme\ rr\ M_4M_4\ g_sg_s$)

In the crossing $Me \times M_1$, 2 hybrids were produced out of 24 crossing attempts, and one of these, Hybrid A, was genetically analyzed. In the crossing $Me \times M_4$, 4 hybrids were produced out of 64 crossing attempts, and one of these, Hybrid C, was genetically analyzed.

These *Me M* hybrids should theoretically segregate out as follows:

Type of ascus	Frequency
2 *Me m*:2 *me M*	1
1 *Me M*:1 *Me m*:1 *me M*:1 *me m*	4
2 *Me M*:2 *me m*	1

The segregation obtained was determined by analysing the ability to ferment melibiose, maltose, and sucrose. In order to demonstrate the existence of complementary gene action, the ability to ferment raffinose was analysed; since cultures which possess only 1 gene (*Me* or *M*) cannot ferment raffinose, positive fermentation tests should be obtained only for cultures containing both *Me* and *M*. Thus, the three types of asci listed above should theoretically ferment raffinose in the following manner:

Type of ascus	Frequency	Raffinose fermentation + —
2 *Me m*:2 *me M*	1	0:4
1 *Me M*:1 *Me m*:1 *me M*:1 *me m*	1	1:3
2 *Me M*:2 *me m*	1	2:2

1–2 day old cultures which had been grown either on 8 % Pilsner wort or on 2 % glucose yeast water were washed and centrifuged and then analysed in Winge fermentometers at 25° C. for their ability to ferment the various sugars. The only exception to this procedure was that, with regard to maltose fermentation, it was found that when the cells had been grown on Pilsner wort prior to the analyses, it was unnecessary to wash them. Two percent maltose, sucrose, and melibiose solutions and 4 % raffinose solutions in yeast water were employed in the fermentation tests. When the cultures had been grown on Pilsner wort, more rapid and clear-cut results with respect to the fermentation of maltose, sucrose, and raffinose were obtained than when the cultures had been grown on glucose.

A. Hybrid A ($Me \times M_1$).

One-day old cultures from glucose of this hybrid fermented maltose after 1 day and melibiose after 2 days, but neither sucrose nor raffinose were fermented during the course of 10 days. The experiment was repeated with 2-day old cultures from wort and the results were then satisfactory: sucrose was fermented after 1 day and raffinose after 3 days. The hybrid is thus able to ferment all four sugars.

The spore germination procent of Hybrid A was 80. Twenty-six asci were obtained in which all 4 spores germinated. Two-day old single-spore cultures from glucose were analysed for their ability to ferment melibiose, maltose, and sucrose. The melibiose analyses gave results completely satisfactory: all asci gave a 2:2 segregation with respect to this sugar after 1–3 days. On the contrary the segregation with respect to maltose and sucrose was not satisfactory, because though several cultures gave strong positive fermentation after 1 day, others gave only weak fermentation after several days. The experiment was then repeated with 2-day-old cultures from wort and the results were completely satisfactory: all the asci gave a 2:2 segregation (strong fermentation already after 1 day), and all maltose fermenters were able to ferment sucrose and vice versa.

Some of the melibiose analyses yielded identical results, regardless of whether glucose or wort had been employed as a substratum.

The raffinose fermentation tests were undertaken with 2-day-old cultures both from glucose and from wort. The cultures which contained both Me and M_1 fermented raffinose: from wort, after 1–4 days and from glucose, after 2–7 days (A-8, however, took 11 days and A-63, 13 days). A-48 (also $Me\,M_1$) from glucose gave a very

weak positive reaction after several weeks (although the hydrolysis, determined by Fehling reaction, was already positive after 1 day); from wort it began to ferment on the eighth day. All the other cultures, which did not contain both *Me* and M_1 (*Me* m_1, *me* m_1, and *me* M_1) were unable to ferment raffinose within 10 days.

Table 4 shows the three types of asci obtained from Hybrid A.

Table 4.

Representative asci from Hybrid A (*Me me* M_1 m_1) showing the type of fermentation results obtained. It will be seen that raffinose is fermented only by those types containing both *Me* and M_1. (Compare Table 7).

Ascus	Culture	Melibiose	Raffinose	Maltose	Sucrose	Genotype
V	A-17	+	+	+	+	*Me* M_1
	-18	—	—	—	—	*me* m_1
	-19	+	—	—	—	*Me* m_1
	-20	—	—	+	+	*me* M_1
VII	A-25	—	—	—	—	*me* m_1
	-26	—	—	—	—	*me* m_1
	-27	+	+	+	+	*Me* M_1
	-28	+	+	+	+	*Me* M_1
IX	A-33	—	—	+	+	*me* M_1
	-34	+	—	—	—	*Me* m_1
	-35	+	—	—	—	*Me* m_1
	-36	—	—	+	+	*me* M_1

Of the 26 asci analysed from Hybrid A, 5 were 2 *Me* m_1:2 *me* M_1 (Type 1), 20 1 *Me* M_1:1 *Me* m_1:1 *me* M_1:1 *me* m_1 (Type 2), and 1 2 *Me* M_1:2 *me* m_1 (Type 3).

The results may be summarized as follows:

A. None of the cultures from Type 1-asci fermented raffinose.

B. One culture (*Me* M_1) from each Type 2-ascus fermented raffinose, while the three others (*Me* m_1, *me* M_1, *me* m_1) were unable to do so.

C. Two cultures (*Me* M_1) from the Type 3-ascus fermented raffinose, while the other two were unable to do so.

The complete segregation results obtained from Hybrid A are summarized in Table 5.

The fermentative ability of 72 cultures (18 of the 26 asci from Hybrid A) was observed for an additional month, because it was expected that the *Me*-cultures could slowly ferment raffinose since they

Table 5.

The raffinose segregation ratios obtained from the progeny of Hybrid A ($Me\ me\ M_1\ m_1$).

Types of asci	Raffinose fermentation ratio	Number of asci: Observed	Number of asci: Theoretical
2 $Me\ m_1$:2 $me\ M_1$	0:4	5	4.3
1 $Me\ M_1$:1 $Me\ m_1$:1 $me\ M_1$:1 $me\ m_1$	1:3	20	17.2
2 $Me\ M_1$:2 $me\ m_1$	2:2	1	4.3

all contained g_s (slow raffinose fermentation through complementary action of $Me + g_s$). Of the 72 cultures studied only 14 contained Me and g_s; 10 of these gave a positive reaction (varying from 11 to 28 days), while the remaining 4 had not yet fermented at the time the experiment was concluded (1 month). As was expected, none of the cultures of the genotypes $M_1\ me\ g_s$ or $m_1\ me\ g_s$ showed, even in this time, signs of being able to ferment raffinose. Table 6 shows that raffinose is rapidly fermented by the complementary action of Me and M_1 and slowly fermented by the complementary action of Me and g_s.

Table 6.

Two representative asci of Hybrid A ($Me\ me\ M_1\ m_1$) showing the rapid and the slow raffinose fermentation brought about by the complementary action of $Me + M_1$ and $Me + g_s$, respectively.

Ascus	Culture No.	Culture Genotype	Days 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
XIII	A-49	$me\ m_1\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	A-50	$Me\ M_1\ g_s$	—	+	++	++	+++	..	..	..	..	..	..	..	..	..	..
	A-51	$me\ M_1\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	A-52	$Me\ m_1\ g_s$	—	—	—	—	—	—	—	—	—	—	+	++	++	++	++
XIV	A-53	$me\ m_1\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	A-54	$me\ M_1\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	A-55	$Me\ m_1\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	+	++
	A-56	$Me\ M_1\ g_s$	—	+	+	++	+++	..	..	..	..	..	..	..	..	..	..

B. Hybrid C ($Me \times M_4$).

1-day-old cultures from glucose of this hybrid fermented maltose, melibiose, and sucrose (1–2 days) but not raffinose. The fermentation with respect to raffinose was then repeated using 2-day-old cultures from Pilsner wort: after 2 days raffinose was fermented. This hybrid,

as well as Hybrid A, is therefore able to ferment raffinose through the complementary action of $Me + M$.

The spore germination procent of Hybrid C was 83. In all, 29 4-spored asci were analyzed for their ability to ferment the various sugars.

The maltose and sucrose fermentation tests were undertaken with 2-day-old cultures from Pilsner wort. The fermentation began rapidly (especially of maltose) and was complete within 24 hours. All 29 asci gave a 2:2 segregation for maltose and sucrose, and the fermenting cultures were able to ferment both sugars.

The melibiose analyses were made with 2-day-old cultures from glucose. All asci gave a clear 2:2 segregation within 2 days.

The raffinose tests were carried out in duplicate with cultures from wort and from glucose. Those from wort which contained both Me and M_4 fermented raffinose after 1–4 days (three after 7 days), while the same cultures from glucose were either much slower or unable to ferment within 10 days. It must be said that also here (as we have already seen in the case of Hybrid A) cultures containing the Me-gene alone begin to ferment after 10 days because of the presence of the g_s gene. It appears therefore that cultures which have been grown on glucose are unsuitable for demonstrating the fermentation of raffinose through the complementary gene action $Me + M_4$. None of the cultures of the genotypes $Me\,m_4$, $me\,m_4$, or $me\,M_4$ were capable of fermenting raffinose within this 10-day period.

The different results obtained with respect to raffinose with glucose-grown and wort-grown cultures are apparently due to a higher concentration of α-glucosidase or to an increased vigor of the cells in wort. The high maltose concentration of Pilsner wort may possibly influence the production of α-glucosidase by those M-yeast cells which are grown on this medium.

Moreover, the slow raffinose fermentation occurring with Me M_4 cultures from wort and particulary from glucose leads to the following possibilities:

1) The presence of liberated sucrose either cannot be demonstrated or can be demonstrated only very slowly, through fermentation, because of an insufficient concentration of the sugar.

2) The unhydrolysed raffinose competes with the liberated sucrose for the α-glucosidase, retarding the hydrolysis of the sucrose, and therefore the fermentation.

The following experiment seems to indicate that raffinose and

sucrose do not compete for the enzyme, but rather that the liberated sucrose is not present in sufficiently high concentration to bring about a rapid fermentation.

Three series of 10 different M_4-cultures (2-days old, from glucose) were analysed for their ability to ferment 2 % sucrose, 1 % sucrose, and 1 % sucrose + 4 % raffinose. After the course of 24 hours all cultures had fermented the 2 % sucrose, but only 2 had begun to ferment the other two media. After 48 hours 5 cultures had fermented the 1 % sucrose and 6 had fermented the mixture. After 4 days, 7 cultures had fermented the 1 % sucrose and 8 had fermented the 1 % sucrose + 4 % raffinose mixture. After 9 days, 8 cultures had fermented the 1 % sucrose and 9 had fermented the 1 % sucrose + 4 % raffinose mixture, and after 11 days all cultures had fermented the 1 % sucrose, and 9 had fermented the mixture. Thus a low concentration of sucrose carries with it a correspondingly low rate of fermentation.

Table 7 shows the three types of asci obtained from Hybrid C, while Table 8 shows that the segregation results from Hybrid C agreed with the theoretically expected, and were essentially similar to those obtained from Hybrid A.

Here again the *Me* g_s cultures from Hybrid C first commenced the fermentation of raffinose after approximately 10 days. Table 9

Table 7.

Representative asci from Hybrid C (*Me me* $M_4 m_4$) showing the type of fermentation results obtained. It will be seen that raffinose is fermented only by those types containing both *Me* and M_4. (Compare Table 4).

Ascus	Culture	Melibiose	Raffinose	Maltose	Sucrose	Genotype
XXI	C-81	—	—	—	—	*me* m_4
	-82	+	—	—	—	*Me* m_4
	-83	—	—	+	+	*me* M_4
	-84	+	+	+	+	*Me* M_4
XXV	C-97	+	—	—	—	*Me* m_4
	-98	—	—	+	+	*me* M_4
	-99	—	—	+	+	*me* M_4
	-100	+	—	—	—	*Me* m_4
XXIX	C-113	—	—	—	—	*me* m_4
	-114	+	+	+	+	*Me* M_4
	-115	—	—	—	—	*me* m_4
	-116	+	+	+	+	*Me* M_4

shows that raffinose is fermented rapidly by the complementary action of $Me + M_4$ and slowly by the complementary action of $Me + g_s$.

Table 8.

The raffinose segregation ratios obtained from the progeny of Hybrid C ($Me\ me\ M_4 m_4$).

Types of asci	Raffinose fermentation ratio	Number of asci: Observed	Theoretical
$2\ Me\ m_4 : 2\ me\ M_4$	0:4	5	4.7
$1\ Me\ M_4 : 1\ Me\ m_4 : 1\ me\ M_4 : 1\ me\ m_4$	1:3	21	19.2
$2\ Me\ M_4 : 2\ me\ m_4$	2:2	3	4.8

Table 9.

Two representative asci of Hybrid C ($Me\ me\ M_4 m_4$) showing the rapid and slow raffinose fermentation brought about by the complementary action of $Me + M_4$ and $Me + g_s$, respectively.

Ascus	Culture No.	Culture Genotype	Days 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
VIII	C-29	$me\ M_4\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	-30	$Me\ m_4\ g_s$	—	—	—	—	—	—	—	—	—	—	+	+	++	++	++
	-31	$me\ M_4\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	-32	$Me\ m_4\ g_s$	—	—	—	—	—	—	—	—	—	—	+	+	++	++	++
XVIII	C-69	$Me\ m_4\ g_s$	—	—	—	—	—	—	—	—	—	—	—	+	++	++	+++
	-70	$me\ m_4\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	-71	$me\ M_4\ g_s$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	-72	$Me\ M_4\ g_s$	—	—	+	++	+++	..	..	..	..	..	..	..	..	..	..

SUMMARY

1. Yeast melibiase (α-galactosidase) hydrolyses the raffinose molecule into galactose and sucrose.
2. The complete hydrolysis of raffinose is brought about not only by the complementary action of the *R*-gene (β-h-fructosidase) and the *Me*-gene (α-galactosidase), but also by the complementary action of the *Me*-gene and the *M*-gene (α-glucosidase). In the second case the melibiase acts first, and the liberated sucrose is then hydrolysed by the α-glucosidase.
3. Yeasts of the genotype *Me M* are able to bring about a 2/3 fermentation of the raffinose molecule.
4. Yeasts of the genotype *Me M* which also contain the *G*-gene

(galactozymase) are able to bring about a complete (3/3) fermentation of the raffinose molecule.

5. Raffinose is fermented more rapidly by *R*-cultures than by *Me M*- or *Me M G*-cultures, and this is presumably due to α-galctosidase effecting a slower rate of hydrolysis than β-h-fructosidase.
6. Hybrids produced by crossing two non-fermenters of raffinose ($Me\ m \times me\ M$) were able to ferment raffinose, and the raffinose segregation of their progeny yielded 0:4, 1:3, and 2:2 ratios in excellent agreement with the theoretically expected results.
7. A slow fermentation of raffinose is possible through the complementary action of the *Me*-gene and the g_s-gene (slow synthesizer of galactozymase).

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