STUDIES ON MICROBIAL METABOLISM OF MYO-INOSITOL

III. Nature and activity of yeasts decomposing myo-inositol

V. N. PARANJAPYE, SARASWATI ROYAN-SUBRAMANIAM,
M. K. SUBRAMANIAM AND J. V. BHAT

[*Fermentation Technology Laboratory and Department of Biochemistry, Indian Institute of Science, Bangalore-12*]

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ABSTRACT

The morphological, cultural and physiological characteristics of an inositol decomposing mutant strain of yeast was compared with its parent strain and two other inositol decomposing species. The results revealed that in several respects clear-cut differences with respect to nitrate reduction, fermentation of sugars, carbon source utilization as well as requirements for vitamins are observable in those that decompose inositol and those that do not. The probable pathway of degradation of inositol by the mutant strain was followed by manometric experiments.

INTRODUCTION

Yeasts, as a group of micro-organisms, were observed to lack the capacity to utilise inositol as the sole source of carbon and energy. Inositol enrichments, suitably modified for the growth of yeasts, also failed to promote their growth [1]. This was somewhat surprising when viewed against the reported requirement by yeasts of inositol not only for the formation of phosphatides but for the processes of cell sythesis and division as well [2]. An investigation on the nature, nutritional requirements, and the physiological behaviour of a few strains capable of attacking inositol was, therefore, undertaken and the results are presented in this paper.

It is of interest to mention here that recently the addition of nicotinamide has been shown to suppress the blank values recorded for inositol assays carried out with *Saccharomyces* and niacin and its coenzymes have thus been linked up in its degradation [3].

MATERIALS AND METHODS

One of the cultures used in this study was an induced mutant strain of *Saccharomyces cerevisiae*, designated BY_2, isolated by Subramanian and Ranganathan[4, 5, 6] from a diploid brewery bottom yeast (BY_1) after exposure to acenaphtene. This culture, unlike its parent strain, was observed to possess the characteristics of a top yeast and possessed two chromosomes of

unequal size. Its poor growth immediately after isolation led to the suggestion that the inequality of chromosomes was the result of a deletion. Studies on its nutritional requirements and physiological characteristics led Prema Bai[7] and Mitra[8] to the conclusion that the mutant was nutritionally less exacting and metabolically less fermentative than its parent culture. Interestingly, the mutant also excreted considerable quantities of riboflavin. For this reason, the latter authors suggested that a chromosomal translocation rather than deletion had taken place in this strain.

This strain, kept under constant observation, was after some years found to produce mutants in the form of elevated buttons on giant colonies. These were isolated from 20-24 day old giant colonies. The variant, on isolation, was observed to be quite distinct from its parent BY_2 and was therefore designated as a new mutant, BY_{2M}. Surprisingly, of the fifty strains of *S. cerevisiae* screened from the collection of Cytogenetics Laboratory of the Department of Biochemistry, only this mutant possessed the ability to adapt and grow in inositol and decompose it. This culture was therefore taken up for the present investigation. For obvious reasons, the strain BY_1 was also included in the study. Of the several marine yeasts examined[9], a strain of *Cryptococcus laurentii* was found to be both pectolytic and capable of oxidising inositol. This culture, with another strain isolated from pectin enrichments, constituted the bulk of cultures taken up for this investigation. Cultures either grown on malt extract, yeast extract media and/or inositol containing medium were used in the experiments.

Morphological studies. For this purpose, the cultures were grown for 24-48 h. on a medium containing 5% malt extract, 0.5% yeast extract and 2.5% agar. The cells were observed under the low powers of a phase contrast microscope. Crystal violet stained preparations were also examined.

Cultural Characters. Giant colonies were also allowed to be formed on malt extract agar and their photographs at various stages of growth were taken.

Reduction of nitrate. Wickerham[10] considers this as an important criterion in the classification of yeasts. Nitrate reduction was followed in the presence individually of two carbon sources, *viz.*, glucose and inositol, each incorporated at 1% concentration along with 0.1% potassium nitrate in a basal salt solution[11]. After 3 and 6 days of incubation, nitrite was detected by its reaction with sulphanilic acid and di-methyl-α-naphtylamin[12]. Ammonia formation was indicated by nesslerization. Negative results were confirmed by determining the presence or otherwise of residual nitrate.

Sugar fermentations. Fermentation or otherwise of carbohydrates was studied in peptone water containing 1% sugar + Andrade's indicator. The method recommended by Hugh and Leifson[13] was also followed to determine whether the strains were metabolically oxidative or fermentative using a

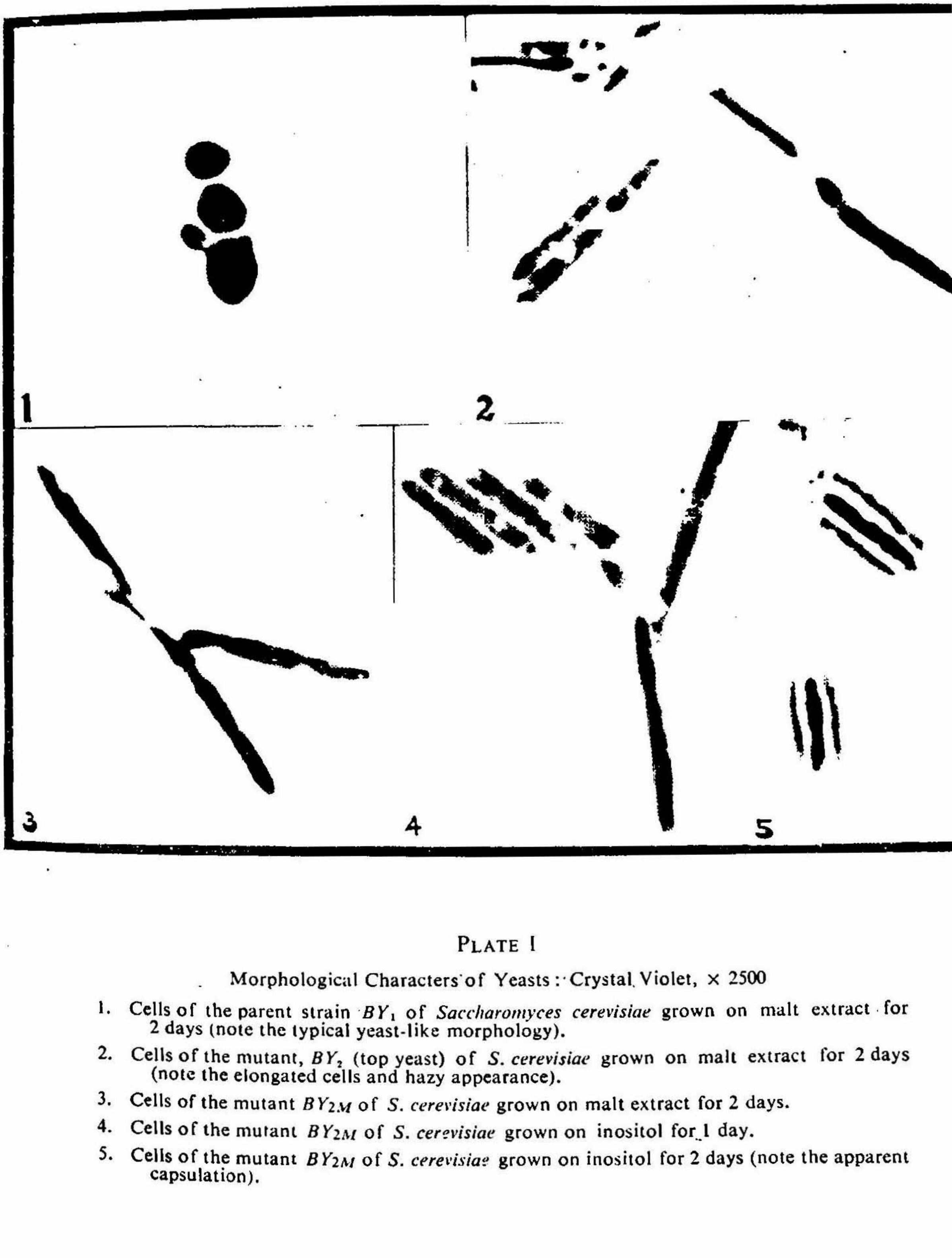

PLATE I

Morphological Characters of Yeasts : Crystal Violet, × 2500

1. Cells of the parent strain BY_1 of *Saccharomyces cerevisiae* grown on malt extract for 2 days (note the typical yeast-like morphology).
2. Cells of the mutant, BY_2 (top yeast) of *S. cerevisiae* grown on malt extract for 2 days (note the elongated cells and hazy appearance).
3. Cells of the mutant BY_{2M} of *S. cerevisiae* grown on malt extract for 2 days.
4. Cells of the mutant BY_{2M} of *S. cerevisiae* grown on inositol for 1 day.
5. Cells of the mutant BY_{2M} of *S. cerevisiae* grown on inositol for 2 days (note the apparent capsulation).

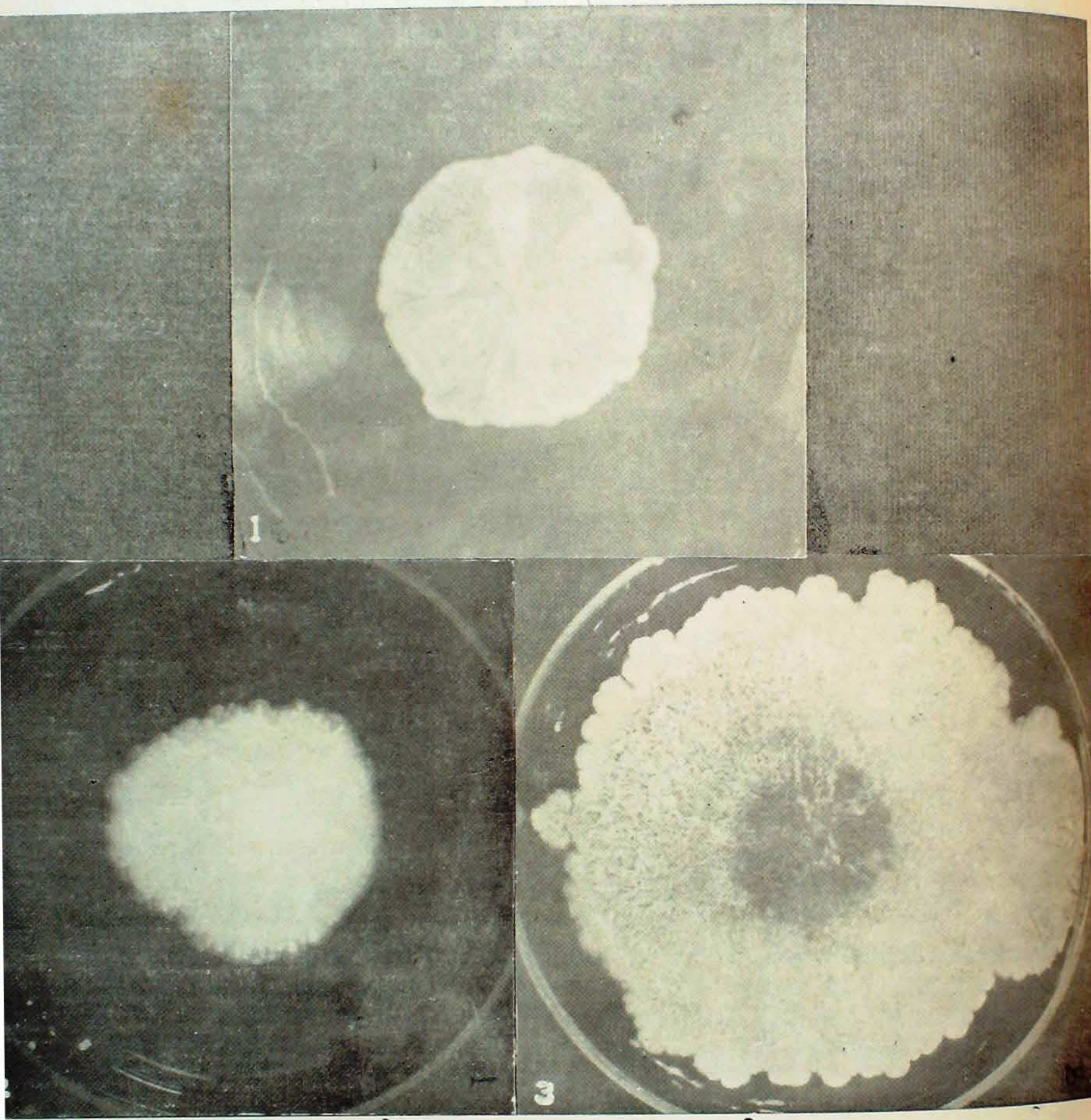

PLATE II

Giant Colonies of Yeasts (20 days Growth on malt extract-yeast extract-agar)

1. Colony of the parent strain BY_1 *S cerevisiae* (3 cm.)
2. Colony of the mutant BY_2 of *S. cerevisiae* (3.5 cm.)
3. Colony of the mutant BY_{M2} of *S. cerevisiae* (8.5 cm.)

medium containing 2 *g* tryptone, 0.5 *g* NaCl, 0.3 *g* K_2HPO_4, and 0.003 *g* bromothymol blue in 1 L. dist. water. After fractional steam sterilization, the cultures were inoculated in two sets of tubes. One set was incubated under aerobic conditions while the other was kept under partially anaerobic conditions using sterile paraffin.

Utilization of carbon sources. This was ascertained by the method described earlier elsewhere[11].

Vitamin requirements. Requirements for vitamins was assessed in two media containing individually glucose and inositol at 1% level with 0.5% ammonium sulphate as the source of nitrogen in an otherwise basal salt solution. The experiments were conducted at room temperature (25-27°*C*) in pyrex test tubes (1.9 × 15 cms.) each containing 10 ml medium. Vitamins were used at the following concentrations : biotin 0.5 γ/ml ; inositol, 100 γ/ml; other vitamins, 0.5 γ/ml. The *pH* was adjusted to 7.0 and the medium was steam sterilized.

The inoculum consisted of a drop of a very dilute suspension of yeasts harvested from malt extract – yeast extract agar slants. With a view to obviate carry-over of nutrients, three serial transfers were made in the same medium and the turbidity was measured in a Bausch and Lomb spectronic analyser after each transfer.

Other methods. The methodology employed for characterization of the products involved in the inositol degradation by yeasts was essentially similar to the one used previously in the study of bacteria[11, 14].

Results

Morphological and cultural characteristics. Morphologically, it was interesting to observe that the parent strain BY_1 was typically a yeast in that its cells were ovoid, small and contained many vacuolar regions besides a dense centre. The mutants, on the other hand, were in striking contrast in that the cells were highly elongated and unevenly stained. However, clear evidences of budding were observed. The cells of BY_{2M} displayed a slightly varied pattern and showed apparent capsulation when grown on inositol, though this seems to be a staining artefact (Plate I).

The colony characteristics (Plate II) of the strains served as the primary criterion for their differentiation. In liquid media, BY_1 was found to grow essentially in the form of a sediment. BY_2, on the other hand, produced a pellicle which on vigorous shaking could be broken up to produce a uniform turbidity. Mutant BY_{2M} also produced a dry, thick pellicle, which was not amenable to be broken up by shaking. Moreover, this mutant was found to produce a highly viscous sediment during the initial stages of growth.

Physiological characteristics

Nitrate reduction. Results of this test revealed that whereas the three inositol decomposing yeasts and the mutant BY_2 could reduce nitrates either to nitrites or to ammonia, the parent strain BY_1 (*S. cerevisiae*) could not do so.

Fermentation of sugars. The fermentative behaviour of strain BY_1 was in accordance with the known specific character. It could ferment with ease most of the common sugars to form gas copiously. BY_2 mutant could produce acid from several sugars but the production of gas was evident only to a slight extent. The mutant BY_{2M}, on the other hand, could produce neither acid nor gas. In general, the inositol decomposing yeasts were oxidative and non-fermentative (I), whereas the parent strain (BY_1) was only fermentative.

Carbon source utilization. The differences in the ability of the yeasts to utilize various substances further highlighted their differences (Table II). BY_1 utilized to a greater or a lesser extent various polysaccharides and mono-di- and trisaccharides, but failed to grow in the presence of lower alcohols, inositol and salts of various organic acids. On the other hand, BY_2 mutant could utilize all the the substrates except the only cyclitol tested, *viz.*, inositol which it could not use even after several transfers. The mutant derived therefrom, *viz.*, BY_{2M} had the ability to adapt and utilize inositol and, subsequently developed the power to utilize also its metabolic products tartrate, oxalate and formate which the other two could not make use of. That tartrate, oxalate and formate represent the breakdown products of inositol has been demonstrated in certain *Xanthomonas* species[11].

Other inositol decomposing yeasts were also found to metabolise most of the substrates except tartrate, oxalate and formate.

Requirements of vitamins. As may be seen from Table III the parent strain of *S. cerevisiae* had an obligatory requirement for thiamin, biotin and inositol. It also required pyridoxin and pantothenic acid during the initial transfer in media deficient in these factors. However, it rapidly acquired the power either to synthesize these substrates or alternatively to by pass their needs. Such results have indeed been observed in the past and have also been explained in the terms of adaptation or of mutation and of selection of mutants under appropriate conditions of growth. Prema Bai[7] also had a similar experience but failed to observe the gradually acquired independance for pyridoxin and pantothenic acid as she did not resort to, during the work, to three serial transfers in the deficient media. The first mutant (BY_2), contrastingly, demanded only biotin whereas the second mutant (BY_{2M}) could do away with it though the growth was luxuriant in the presence of some yeast extract.

Like the BY_{2M}, the other two inositol decomposing yeasts did not grow well in media devoid of either thiamine or yeast extract although the strains were not exacting to any vitamins.

TABLE I

Fermentation of sugars by yeasts

Culture and isolate No.	Glucose	Galac-tose	Fructose	Lactose	Maltose	Sucrose	Raffi-nose	Inositol	Grown under anaerobic conditions (Fermentative)	Grown under aerobic conditions (Oxidative)
Saccharomyces cerivisiae BY_1*	†	†	†	–	†	†	†	–	+	–
do BY_2*	+*a*	+*a*	+*a*	–	+	+*a*	+*a*	–	–	+
do BY_{2M}*	–	–	–	–	–	–	–	–	–	+
Unidentified 25*	+		+	–	–+	–+	–+	–	–	+
*Cryptococcus laurentii**	+	–	+	–	–	–	–	–	–	+
S. cerevisiae BY_{2M}**	–	–	–	–	–	–	–	–	–	+
Unidentified 25**	+	–	+	–	+	+	–	–	–	+
*Cryptococcus laurentii***	+	–	+	–	+	+	+	–	–	+
Uninoculated control	–	–	–	–	–	–	–	–	–	–

* Cultures adapted to malt extract-yeast extract. ** Cultures adapted to inositol. –+Slight production of acid.
+Production of acid. +*a* Slight production of gas after 15 days. † Production of gas. –Absence of formation of acid and/or gas

Utilization of various carbon sources by yeasts
(After incubation for 8 days at 25°-27°C.)

Substrate		*Saccharomyces cerevisiae* BY_1*	*Saccharomyces cerevisiae* BY_2*	*Saccharomyces cerevisiae* BY_{2M}*	Unidentified [25*]	*Cryptococcus laurentii**	*Saccharomyces cerevisiae* BY_{7M}**	Unidentified 25**	*Cryptococcus laurentii***
Control	.....	–	–	–	–	–	–	–	–
Starch	.....	– +	+	3 + P	+	+	– +	– +	+
Dextrin	.....	– +	+	2 + P	– +	+	2 + P	– +	+
Raffinose	.....	3 +	4 + P	3 + P	2 +	2 +	3 + P	3 +	+
Maltose	.....	4 +	4 + P	3 + P	3 +	3 +	3 + P	3 +	2 +
Lactose	.....	–	– +	2 + P	2 +	+	3 + P	3 +	+
Sucrose	.....	3 +	4 + P	3 + P	3 +	3 +	3 + P	3 +	2 +
Glucose	.....	3 +	4 + P	3 + P	3 +	3 +	3 + P	4 +	3 +
Galactose	.....	4 +	4 + P	3 + P	3 +	+	3 + P	2 +	2 +
Fructose	.....	4 +	4 + P	3 + P	2 +	2 +	3 + P	3 +	2 +
Inositol	.....	–	–	3 + P	2 +	2 +	3 + P	3 +	3 +
Mannitol	.....	–	4 + P	2 + P	+	2 +	2 + P	2 +	+
Dulcitol	.....	–	4 + P	+ P	+	– +	– +	+	+
Sorbitol	.....	–	4 + P	3 + P	3 +	3 +	3 + P	3 +	3 +
Glycerol	.....	–	4 + P	2 + P	2 +	2 +	3 + P	2 +	+
Ethyl alcohol	.....	–	2 + P	2 + P	+	+	2 + P	– +	+
Methyl alcohol	.....	–	2 + P	2 + P	+	2 +	3 + P	+	+
Citrate	.....	–	3 + Alk. P	2 + Alk. P	2 + Alk.	+ Alk.	2 + Alk. P	2 + Alk.	2 + Alk.
Succinate	.....	–	3 + Alk. P	3 + Alk. P	2 + Alk.	2 + Alk.	3 + Alk. P	2 + Alk.	2 + Alk.
Fumarate	.....	–	3 + Alk. P	3 + Alk. P	2 + Alk.	2 + Alk.	3 + Alk. P	2 + Alk.	2 + Alk.
Lactate	.....	–	2 + Alk. P	2 + Alk. P	2 + Alk.	+ Alk.	3 + Alk. F	2 + Alk.	2 + Alk.
Acetate	.....	–	2 + Alk. P	2 + Alk. P	2 + Alk.	+ Alk.	2 + Alk P	+ Alk.	– +
Tartrate	.....	–	–	2 + Alk. P	2 + Alk.	–	+ Alk. P	2 + Alk.	–
Oxalate	.....	–	–	–	–	–	2 + Alk. P	–	–
Formate	.....	–	–	–	+ Alk.	–	+ Alk. P	–	–

* Cultures adapted to malt extract-yeast extract, ** Cultures adapted to inositol. — no growth ; —+ slight growth + poor growth ; 2+ moderate growth ; 3+ good growth ; 4+ luxuriant growth ; Alk. —alkalinity produced ; P—pellicle formed.

TABLE III

Requirements of vitamins by yeasts (in glucose medium)
(Growth expressed as 100—% transmission after 7 days.)

Medium	*Saccharomyces cerevisiae* (BY_1)			*Saccharomyces cerevisiae* (BY_2)			*Saccharomyces cerevisiae* (BY_{2M})*			Unindentified (25)			*Cryptococcus laurentii*		
	Transfers			Transfers			Transfers			Transfers			Transfers		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
No vitamins	4	5	5	11	5	3	Poor	Poor	Poor	15	18	22	14	16	19
All vitamins	88	88	94	76	87	97	Good	Good	Good	43	57	61	33	45	52
– Thiamine	0	5	4	78	90	96	,,	,,	,,	18	19	20	14	17	21
– Riboflavin	88	88	93	78	90	95	,,	,,	,,	33	53	60	31	43	53
– Pyridoxin	24	66	94	87	92	92	,,	,,	,,	36	55	57	28	47	52
– Niacin	88	88	90	87	93	96	,,	,,	,,	36	54	58	30	45	50
– Pantothenic acid	37	88	94	86	94	92	,,	,,	,,	37	57	60	34	42	49
– Pteroyl glutamic acid	88	87	90	90	94	95	,,	,,	,,	36	55	58	29	44	47
– Biotin	0	0	0	15	8	3	,,	,,	,,	36	55	60	29	43	45
– Inositol	0	0	0	88	93	97	,,	,,	,,	34	54	58	32	42	42
– para-Amino benzoic acid	88	88	94	88	92	95	,,	,,	,,	34	51	57	30	41	41

* Turbidimetric readings could not be taken since the culture grew in the form of a pellicle.

TABLE III—*concld.*

Requirements of vitamins by yeasts in (in inositol medium)

(Growth expressed as 100% transmission after 7 days)

Medium	*Saccharomyces cerevisiae* (BY2M)*			Unidentified (25)			*Cryptococcus laurentii*		
	Transfers			Transfers			Transfers		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
No vitamins	Poor	Poor	Poor	16	18	19	10	13	15
All vitamins	Good	Good	Good	32	53	64	23	42	52
– Thiamin	,,	,,	,,	28	30	25	18	23	20
– Riboflavin	,,	,,	,,	32	48	61	22	42	50
– Pyridoxin	,,	,,	,,	32	47	62	23	38	51
– Niacin	,,	,,	,,	33	47	65	23	42	50
– Pantothenic acid	,,	,,	,,	33	45	67	24	42	68
– Pteroyl glutamic acid	,,	,,	,,	32	47	63	23	36	49
– Biotin	,,	,,	,,	28	43	62	23	40	51
– para-Aminobenzoic acid	,,	,,	,,	33	53	62	22	42	50

*Turbidimetric readings could not be taken since the culture grew in the form of a pellicle.

Inositol degradation by yeasts. Certain attempts were made to charaeterise the pathway of inositol degradation by the mutant BY_{2M}. During the initial stages of growth, the mutant was found to produce a highly viscous sedimental growth. On partial purification of this (alcohol precipitation and dialysis), it was found to contain less than 1 % of nucleic acids, less than 10 % of the proteins and less than 20 % of anthrone reactable substance. Its acid hydrolysate contained only negligible amounts of phosphorus and only traces of glucose and galactose. It gave a sharp staining reaction with toluidene blue not unlike the one given by pure chondriotin sulphate and it could reasonably be inferred that it was a mucopolysaccharide. However, since this substance as produced during the initial periods of growth on other substrates as well (and was not a characteristic specific to inositol degradation) no further attempts were made to characterise it.

The culture was extremely difficult to handle and gave very inconsistant results. Several repetitions were hence carried out before drawing conclusions of any kind. It showed a maximum oxidative activity only during the initial periods of growth when it produced maximum mucopolysaccharide. The activity was rapidly lost along with viscosity if the culture was allowed to grow even for a slightly longer period For maximal activity the culture was grown for 18-20 hr. at room temperature on a mechanical rotary shaker. Under these conditions, the inositol adapted whole cells could rapidly take up oxygen from inositol, 2-keto-inositol, glucose, pyruvate, tartrate, oxalate and formate. They could not oxidise glucuronate nor was this acid found in the end products. Similarly, tartrate, oxalate and formate oxidising enzymes were also found in glucose adapted cells. These enzymes appear to be constitutively present in the cells and the acids could not be detected in the end products from inositol decomposition.

Sodium arsenite, at a concentration of $4 \times 10^{-3} M$ inhibited the oxygen uptake from inositol and 2-keto-inositol by about 40 %. Under these conditions, accumulation of pyruvic and α-ketoglutaric acid was observed. Sodium fluoride (5×10^{-2} M) was also found to inhibit the inositol oxidation to a considerable extent. Various attempts to detect the uptake of inorganic phosphorus during inositol degradation by whole cells yielded negative results.

Discussion

Mutations vis-a-vis microbial physiology. One of the most striking effects of mutation was on the morphology of yeasts in the sense that both the mutants were elongated and exhibited an irregular staining Such effects of changes in chromosomal constitution on morphology have indeed been known earlier [15].

The induced mutants seem to have lost their power of fermenting sugars. A possible reason for this may be their failure to elaborate suitable protein-phosphate binding enzymes which would naturally hinder the formation of

flavoproteins. The observation that one of the mutants (BY_2) excretes riboflavin seems to support the hypothesis. Thus, a loss of glycolytic enzymes occurs. Instead, the citric acid cycle, the pentose cycle and the glyoxylic acid cycle enzymes seem to become operative as is evidenced by the ease with which the mutants decompose the intermediary metabolites involved in these processes. Much further work would be necessary to clearly elucidate the effect of mutations on the biochemical processes involved in the metabolism.

The mutation also affects the vitamin requirement of the yeasts. The mutants became progressively more versatile in the sense that they develop the ability to decompose more and more carbon sources needing lesser and lesser vitamins for the process. Thus, the conclusions drawn by Prema Bai and Mitra seem to be correct and the first mutation seems to be a result of chromosomal translocation rather than a deletion.

Inositol decomposing yeasts. The three yeasts that were found to be able to metabolise inositol were all non-fermentative (oxidative) and more or less non-exacting in so far as the vitamin requirements are considered. Whether this is a characteristic shared by all inositol decomposing yeasts can be found out only after a detailed study of a large number of cultures is carried out.

The mechanism of inositol degradation by yeasts is far from understood. The mutant (BY_{2M}) of *S. cerevisiae* seems so follow a process essentially similar to the one described in the case of *Arthrobacter citreus*[14]. However, there seem to be some differences when one takes into consideration the effect of NaF and the ability of only the inositol adapted cells to grow with oxalate, tartrate and formate as sole carbon sources.

REFERENCES

1. Paranjapye, V. N. and Bhat, J. V. — *Indian J. Med. Res.*, 1961, **49**, 688.
2. Postarnak, Th. ... ... — "Les Cyclitols", Herman, Paris, 1962.
3. Braekkan, O. R, and Boge, G. ... — *Nature*, 1963, **198**, 585.
4. Subramaniam, M. K. and Ranganathan, B. — *Nature*, 1946, **157**, 49.
5. *Idem.* ... ... — *Science and Culture.* 1947, **13**, 102.
6. *Idem.* ... ... — *Proc. Natl. Inst. Sci.* (India), 1948, **14**, 315.
7. Prema Bai, M. ... ... — *Curr. Sci.* 1947, **16**, 316.
8. Mitra, K. K. ... ... — "Mutant Strains of Yeasts and their Industrial Importance". Ph.D. Thesis, University of Bombay, 1950.
9. Billmoria, M. H. ... ... — Unpublished.
10. Wickerham, L. J. ... ... — U. S. Dept. Agri. Tech. Bull. 1951, **1029**.
11. Paranjapye, V. N. and Bhat, J. V. — *J. Indian Inst. of Sci.*, 1961, **43**, 104.
12. Conn, H. J. ... ... — "Manual of Microbiological Methods", McGraw Hill, New York, 1957.
13. Hugh, R. and Leifson, E. ... — *J. Bacteriol.*, 1953, **66**, 24.
14. Paranjapye, V. N. and Bhat, J. V. — *Proc. Nat. Inst. Sci.*, (India) 1963, **28**, 532.
15. Beadle, G. W. ... ... — *Currents in Biochem. Res.* 1946, **1**.