The location and identification of the enzyme system responsible for the fermentation of isomaltose in Candida utilis
by John Edward Robbins

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
In this investigation isomaltase was isolated and identified as the enzyme which was responsible for the fermentation of isomaltose in the yeast strain, Candida utilis. The enzyme was located primarily in the cell membranes and small amounts were found in the interior of the cell. The enzyme activity of the membranes was far greater than that of the intracellular sap. Approximately eighty per cent of the enzyme activity was extracted from the membranes by pH adjustment indicating that the enzyme was held by virtue of electrical charge. In addition, evidence is presented that there are two molecular forms of isomaltase present. There is also evidence that the yeast cells could utilize panose as well as isomaltose and in fact they were able to grow well even when isomaltose and panose were the sole sources of carbohydrate. However, there was no evidence under the conditions used where the yeast cells could utilize isomaltotriose and higher homologues.

THE LOCATION AND IDENTIFICATION OF THE ENZYME SYSTEM RESPONSIBLE FOR THE FERMENTATION OF ISOMALTOSE IN *CANDIDA UTILIS*

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ABSTRACT

In this investigation isomaltase was isolated and identified as the enzyme which was responsible for the fermentation of isomaltose in the yeast strain, Candida utilis. The enzyme was located primarily in the cell membranes and small amounts were found in the interior of the cell. The enzyme activity of the membranes was far greater than that of the intracellular sap. Approximately eighty per cent of the enzyme activity was extracted from the membranes by pH adjustment indicating that the enzyme was held by virtue of electrical charge. In addition, evidence is presented that there are two molecular forms of isomaltase present. There is also evidence that the yeast cells could utilize panose as well as isomaltose and in fact they were able to grow well even when isomaltose and panose were the sole sources of carbohydrate. However, there was no evidence under the conditions used where the yeast cells could utilize isomaltotriose and higher homologues.

INTRODUCTION

Isomaltose has been considered a nonfermentable sugar for many years, yet, in the past ten years several investigators (9, 15, 17, 24) have found that isomaltose dissappears from wort used as yeast nutrient. None of these investigators have isolated the enzyme responsible for this utilization. K. J. Goering and M. J. Houle observed this phenomenon several times while conducting research on fermentations by Candida utilis, N.R.R.L. Y900.* K. J. Goering (24) also observed that fermentation stopped for a period of time and then continued once again in a culture of Saccharomyces cerevisae in which glucose, maltose and isomaltose were used as the source of carbohydrate. The maltose and glucose were fermented first and isomaltose last. This suggested that the fermentation of isomaltose required an adaptation period. This was also the conclusion of Okada (9), who found similar evidence of isomaltose fermentation by Shizosaccharomyces pombe and, although he did not isolate the enzyme, he suggested the fermentation was through glucose produced by an extracellular isomaltase, whose production was induced by contact with isomaltose.

The objective of this investigation was to locate (i.e. to find in which subcellular fraction the enzyme existed) and to isolate the system responsible for isomaltose fermentation in Candida utilis. Some investigators believe that certain cells require permeases for the uptake of some sugars and other nutrients (5, 21). Other investigators feel that perme-

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*N.R.R.L. - Northern Regional Research Laboratories, Yeast strain 900, Peoria, Illinois

ases are not required at all and that the uptake of nutrients is more a passive phenomenon than an active one (16, 18, 21). A great deal of time was spent in the early part of this investigation in designing experiments and trying to prepare uniformly labeled isomaltose and higher homologues in an effort to isolate and follow the activity of a permease.

MATERIALS AND METHODS USED

Preparation of Isomaltose

Of the several methods for the preparation of isomaltose described in the literature, the method of Hultin and Nordstrom (11) and Jeanes et al. (13) was chosen because it resulted in high yields of isomaltose. Even after several trials using this method, the reported yields could not be obtained. Other methods giving yields of ten per cent (4, 19) were used because the previous method was too time consuming and the yields obtained were not appreciably better than ten per cent.

Acid Hydrolysis of Dextran

A bacterial dextran produced by Leuconostoc mesenteroides, N.R.R.L. B512,* from sucrose was obtained by growing the organism in the following Medium:

Sucrose	10 grams / 100 ml of H_2O
K_2HPO_4	0.05 gram / 100 ml of H_2O
Yeast Extract	0.25 gram / 100 ml of H_2O
$MgSO_4 \cdot 7H_2O$	0.02 gram / 100 ml of H_2O
NaCl	0.10 gram / 100 ml of H_2O

The production of the dextran may be represented simple by the following equation:

$$n \text{ Sucrose} + \text{Dextransucrase} \longrightarrow n \text{ Fructose} + \text{Glucose}_n.$$

This dextran reportedly has 95% α (1,6) linkages with small amounts of

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*Northern Regional Research Laboratories, Bacteria 512, Peoria, Illinois

$\alpha(1,4)$ and $\alpha(1,3)$.

Fifty-three grams of dextran were dissolved in 4.46 liters of water by heating. This solution was cooled and 540 milliliters of 3N sulfuric acid was added. The solution was stirred constantly for seven hours at 90° C., cooled, and the pH adjusted to 6.0 by slowly adding 3N sodium hydroxid with agitation. Isomaltose was produced in approximately fifteen per cent yield.

Conversion of Maltose to Isomaltose and Panose.

Isomaltose was also successfully prepared by the method of Pazur (19), which converts maltose to isomaltose and panose by means of a fungal transglucosylase.

Four hundred grams of B-maltose dissolved in 2.0 liters of water were added to one liter of a Takamine clarase solution containing ten grams of the clarase. This solution was incubated at 30° C. for 72 hours and then analyzed for the sugars present by paper chromatography. This analysis indicated a minimum concentration of maltose and a high concentration of glucose, isomaltose, and panose. The solution was boiled five minutes to inactivate the enzyme followed by the addition of fifty grams of bakers' yeast suspended in one hundred milliliters of water. After 24 hours the glucose and maltose were removed by fermentation leaving the isomaltose and paose in the reaction mixture.

Separation of Isomaltose

Although completely pure isomaltose was not obtained, column chromatography, using a carbon-celite column containing equal amounts of each

by weight, proved more satisfactory than Sephadex-G-25 for separating isomaltose from sugar mixtures. A column of Darco-G-60 and Celite-535 (7.5 x 95 cm.) was prepared by mixing the absorbents in water and pouring this slurry into the column, which was plugged with glass wool. Concentrated hydrochloric acid was allowed to drip slowly through the column for deactivation of the carbon and to remove traces of iron and the alkaline ash from the celite. Water was then passed through the column until the effluent was neutral.

The sugars were put on the column in aqueous solutions in concentrations that were not less than 0.25 per cent nor greater than five per cent. For high efficiency not more than one gram of sugar mixture was added for each 150 c.c. of carbon-celite. Successive elutions were made with four liters of water, five liters of five per cent ethanol and six liters of fifteen per cent ethanol. Fifteen one liter fractions were collected. A chromatogram of these various fractions is depicted in Figure 1. The separation of isomaltose and panose is depicted in Figure 2, (23).

Pure isomaltose was obtained in amounts of 20-30 milligrams using paper chromatography. Whatman 3 mm paper (22 x 35 cm.) was painted with a solution of isomaltose and higher homologues witha a small paint brush on a line 6 cm. from the top of the paper. Repeated applications were made until the residue on the paper looked almost crystalline. The sheet of paper was then placed in a chromatocabinet for 24 hours. Strips were cut from the edges of the chromatogram and dipped into C-D-1, (see page 9) and then heated to locate the bands of the different sugars. The strip

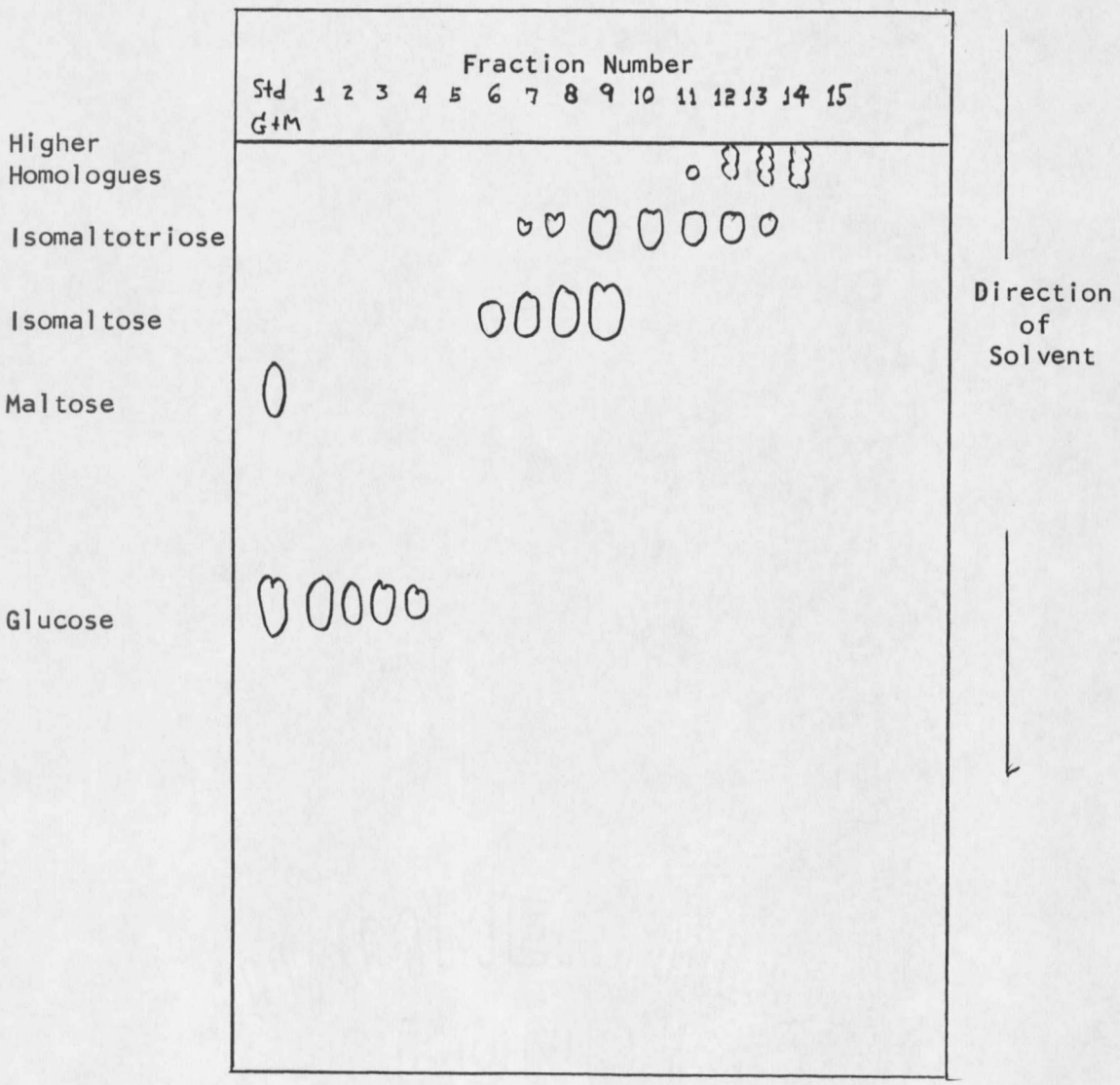

Figure 1. Chromatogram of 1000 ml. Fractions Obtained from Acid Hydrolysis of Dextran

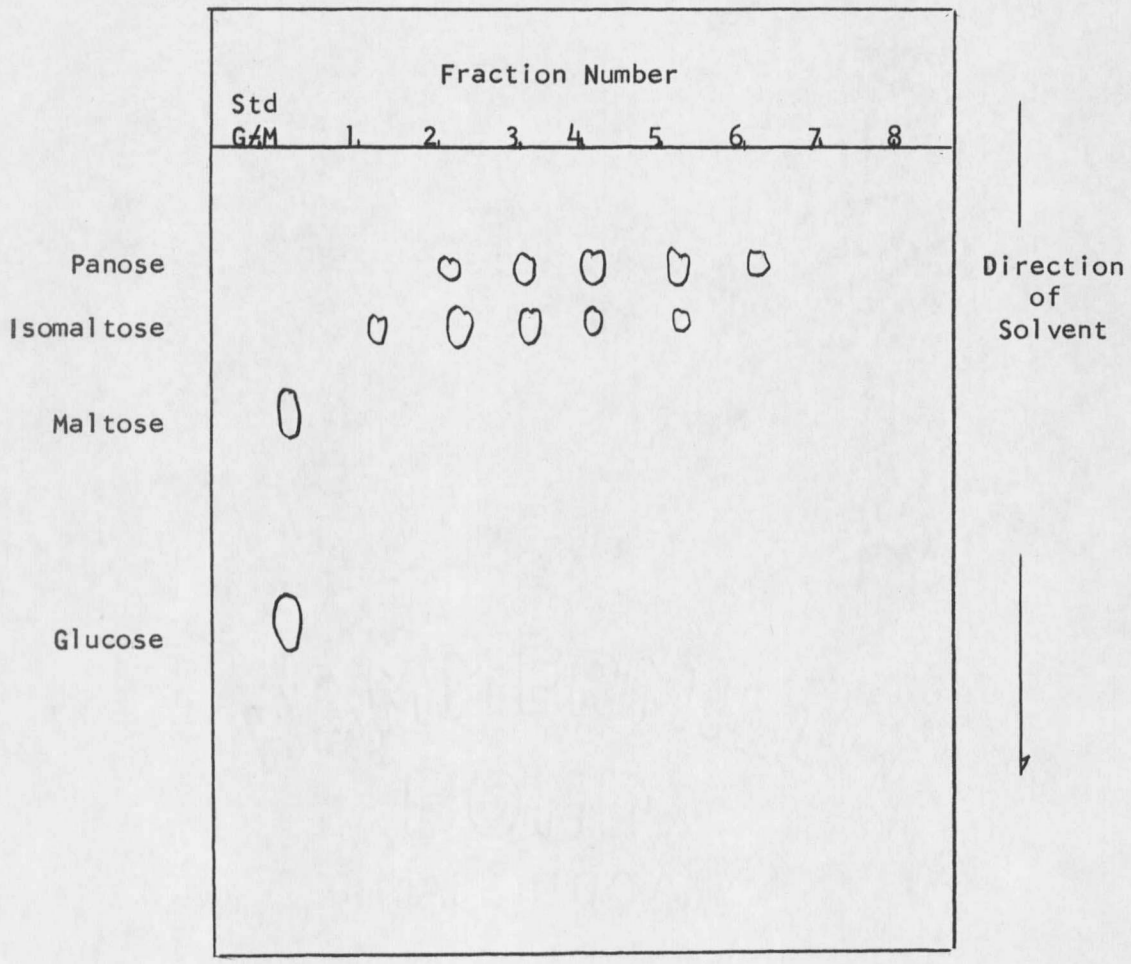

Figure 2. Chromatogram of the Fractions from the Maltose Conversion with Clarase

containing isomaltose was cut out and the isomaltose eluted with water. This solution was concentrated by heating at 80° C. under vacuum.

Sugar Analyzes

Sugar mixtures, such as the products from the acid hydrolysis of the dextran, were analyzed by quantitative paper chromatography (4). The sugars were located on the chromatogram by developing the spots of the separated unknown and a mixture of known sugars with C-D-1. The spots on the strip containing the unknown were then used to locate the spots on an undeveloped strip of the unknown. These spots were eluted with water, diluted to fifty milliliters, and analyzed by the phenol-sulfuric acid method of Dubois and Gilles (6). The mixture from the acid hydrolysis of dextran, after the glucose was removed, contained forty per cent isomaltose, twenty per cent isomaltotriose and forty per cent higher homologues calculated on the basis of micromoles of glucose. On the basis of reducing sugar, isomaltose accounted for 58 per cent, isomaltotriose 19 per cent, and the higher homologues 23 per cent. Unless otherwise stated this solution was used as the substrate solution in this investigation. Isomaltose accounted for seventy per cent of the micromoles of glucose in the panose-isomaltose mixture. An example of these calculations follows.

Phenol-sulfuric acid analysis gave:

Isomaltose	- 20 μg of glucose
Isomaltotriose	- 10 μg of glucose
Higher homologues	- 20 μg of glucose

Isomaltose - 20 x 100 / 50 = 40%

20 μg of glucose = 0.11 μ moles of glucose

20 μg of isomaltose = 0.055 μ moles of isomaltose

10 μg of isomaltotriose = 0.018 μ moles of isomaltotriose

20 μg of isomaltopentaose = 0.022 μ moles of isomaltopentaose

Total μ moles of reducing sugar = 0.095 μ moles

per cent reducing sugar = 55 x 100 / 95 = 58 per cent.

A modification of the method of Sumner (20) was used to analyze for reducing sugars. Since the reagent will deteriorate with time it was necessary to run a standard with each test as a precaution. The standard curve was based on maltose.

Chromatogram Solvent System

The solvent system used to develop all the chromatograms in this investigation was a mixture of butanol, pyridine, and water in a volume to volume ratio of 6:4:3 respectively unless described otherwise. Whatman number one filter paper was used for all chromatograms. Probably due to more uniform temperature and atmosphere saturation a great improvement in the separations of the sugars occurred when a new Research Specialties chromatocabinet was used.

Carbohydrate Color Developer

The carbohydrate color developer used was that of Gorden et al. (8). The chromatograms were dipped in C-D-1, dried, and then heated with a Master heat blower to develop the color of the spots. The color of the

spots in conjunction with the R_{gl}* values were used for identification of the various sugars.

Yeast Nutrient

The nutrient for the yeast cultures contained the following substances per gram of sugar in one hundred milliliters of nutrient:

Urea	0.12 gram
$Ca(H_2PO_4)_2$	0.03 gram
KCl	0.01 gram
$MgSO_4 \cdot 7H_2O$	0.01 gram.

The nutrient was sterilized by autoclaving for fifteen minutes at 15 psi, then cooled to room temperature and the pH adjusted to 4.5 with 1NHCl. The type of sugar was varied in different experiments but otherwise all nutrient was prepared in the above manner. The cultures were incubated aerobically in a thermostated shaker at 20°C. In order to remove the cells from the media for the various experiments throughout this investigation, the cultures were centrifuged at 4,000 x g.

Identification of Isomaltose

Isomaltose was identified by paper chromatography and optical rotation. A value for the migration ratio of isomaltose to glucose, R_{gl}, was given by Whistler (4) as 0.43 in a solvent mixture of ethyl acetate, pyridine, and water in a volume ratio of 8:2:1 respectively. The sugar

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*R_{gl} is the ratio of migration distance of a sugar as compared to the migration distance of glucose.

that was tentatively identified as isomaltose gave an identical R_{gl} value when the same solvent system was used. The R_{gl} values in two solvent systems for maltose and isomaltose are given in Table I. The development time was 24 hours and the temperature was 30° C.

TABLE I

R_{gl} Values of Maltose and Isomaltose

Solvents	8 Ethyl Acetate 2 Pyridine 1 Water		6 Butanol 4 Pyridine 3 Water
	Lit. Value	Obs. Value	
Glucose	1.00	1.00	1.00
Maltose	0.65	0.66	0.68
Isomaltose	0.43	0.43	0.47

W. Pigman (3) gave a specific rotation value of +120° for isomaltose. A specific rotation of +120.5° was obtained for the sugar identified as isomaltose which in conjunction with the chromatographic identification was considered ample proof of identity. A rigorous identification of panose was considered unnecessary since Pazur (19) identified isomaltose and panose as being the only products of the reaction. The positions of the isomaltose and panose spots on the chromatograms corresponded very well with these shown by Pazur.

EXPERIMENTS, RESULTS, AND DISCUSSION

The Ability of Candida utilis to Utilize Various Carbohydrates

A comparison of the utilization of glucose and maltose was made over the range of zero to one hundred per cent of glucose. Samples were withdrawn hourly from the cultures for four hours and the sugar concentrations were determined by paper chromatography (4). The results are given in Table II.

The data obtained definitely showed that the yeast used glucose more readily than maltose. It was interesting to note that when the maltose concentration was decidely larger than that of glucose, the maltose was utilized, or disappeared, more rapidly; when the ratio of glucose to maltose was one or larger, glucose was utilized more rapidly. In the case of the one hundred per cent glucose culture, maltose was produced. Glucose was produced in the one hundred per cent maltose culture. This indicated an equilibrium which might be caused by the presence of an extracellular maltase.

Three separate cultures were prepared to compare the ability of the yeast to utilize isomaltose and panose with respect to maltose, and to see if it would be possible for the yeast to utilize dextran. Since an adaptation period was anticipated, 0.5 per cent maltose was included in the isomaltose-panose and one per cent maltose in the dextran culture. The total carbohydrate concentration was approximately two per cent by weight. A culture containing one per cent maltose was included.

The wet cell weight of these cultures was determined at the end of

TABLE II

Ratio of Glucose : Maltose with Time

%G - %M[1]	1st hour ratio	2nd hour ratio	3rd hour ratio	4th hour ratio
10 - 90	1.7 : 8.3	1.2 : 8.8	1.0 : 1.0	C. U.[2]
20 - 80	3.1 : 6.9	1.9 : 8.1	2.2: 7.8	C. U.
30 - 70	4.3 : 5.7	1.9 : 8.1	1.0 : 1.0	C. U.
40 - 60	5.2 : 4.8	3.4 : 6.6	4.0 : 6.0	C. U.
50 - 50	5.5 : 4.5	4.4 : 5.6	3.5 : 6.5	C. U.
60 - 40	4.2 : 5.8	4.5 : 5.5	3.3 : 6.7	C. U.
70 - 30	2.5 : 7.5	5.7 : 4.3	3.8 : 6.2	C. U.
80 - 20	7.2 : 2.8	4.9 : 5.1	6.4 : 3.5	C. U.
90 - 10	8.3 : 1.7	6.7 : 3.3	4.6 : 5.4	C. U.
100 - 0	9.2 : 0.8	6.7 : 3.3	5.1 : 4.9	C. U.
0 - 100	.0 : 10	1.5 : 8.5	3.2 : 6.8	C. U.

[1] % G - % M -- % Glucose to % Maltose

[2] C. U. -- the sugars were completely utilized