Preparation of Melibiase-Invertase for Sugar Beet Molasses Assay of Raffinose

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Introduction

The quantitative determination of sucrose in the presence of raffinose, in beet sugar factory materials, is considered most reliable when based upon methods involving the use of the enzymes invertase and melibiase. A commercial source of the latter enzyme could not be found in recent years.

Since suitable brewer's yeast, bottom fermenting, could not be obtained in quantity, a procedure for culturing a selected brewer's yeast in the laboratory was developed. The source of this selected yeast, and the harvest of its cultured yeast cells are described.

Extraction of invertase-melibiase from the yeast cells was performed according to the method described by Reynolds (2)[2].

Description of Yeast

A pure culture of the yeast *Saccharomyces carlsbergensis,* collection number C-126, was obtained from the Department of Food Science, University of California at Davis. This yeast was selected because of its ability to produce melibiase-invertase.

Procedures

The preparation of melibiase was begun with yeast inoculation into a growth medium. The yeast was cultured about four days and then harvested by filtration and the filtrate discarded. The yeast cells were autolysed in toluene. The autolysate was filtered and rinsed. The residue was discarded and the filtrate containing the enzymes was purified and concentrated by ultrafiltration. The melibiase concentrate was vacuum dried and the activity determined.

Laboratory Culture of Yeast

The yeast *S. carlsbergensis* was cultured in a 20 liter Pyrex bottle which contained 10½ liters of medium and yeast. The culture bottles, medium, and all accessories which could be the source of microbial contamination, were sterilized either by hot, dry air or by autoclaving. A modified Sabouraud medium at pH 4.8 and 7.6 rds was used for yeast cultivation.

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[2] Numbers in parentheses refer to literature cited.

Table 1.—Yeast Growth Medium.

Constituent	Gms./Liter of Basal Nutrient
Sucrose	40.0
Raffinose	10.0
Dextrose	10.0
Polypeptone (Difco)	10.0
Yeast Extract (Difco)	8.0
Betalin (Lilly) 1:100	10.0 ml.
Basal Nutrient	**Gms/Liter, in Water**
$Ca(NO_3)2 \cdot 4H_2O$	1.12
KH_2PO_4	2.45
$MgSO_4 \cdot 7H_2O$	3.70
Micro-Enrichment Solution	**Mg/Liter, in Water**
(Use 1 ml. for each liter of Basal Nutrient)	
H_3BO_3	600
$MnCl_2 \cdot 4H_2O$	400
$ZnSO_4$	50
$H_2MoO_4 \cdot 4H_2O$	20
$Cu(Ac)_2$	20
$Co(Ac)_2$	20
$FeSO_4$	7,000

Balab No. 613 antifoam is added ½ drop per liter of growth medium nutrient.

To reduce the time required for maximum vigorous growth in the large culture, a "mother culture" was first prepared in a one liter flask. About 500 ml. of growth medium were used in the "mother culture" which was inoculated with a loop of the yeast. The mother culture was incubated for 24 hours at room temperature and then carefully poured into the 20 liter culture bottle together with 10 liters of medium. Then all the accessories were attached to the culture bottle and aeration was begun.

After eight hours of growth the nutrient supplement was fed to the culture at a rate which maintained the culture between pH 3.5 and pH 4.8 and the sugar concentration above 4.0 rds.

Table 2.—Nutrient Supplement: (pH 4.8, rds 20.8).

Sucrose	440 g.
Raffinose	200 g.
Nutrient solution	3 liters

The practical maximum yeast cell development usually required four days. Twelve hours before the yeast cells were to be harvested, aeration was stopped to allow the cells to settle thus facilitating the harvest.

Filtration of Yeast Cells from Growth Media

When the yeast cells were ready for harvest they were separated from the growth medium by vacuum filtration on a Buchner funnel. Filtration was simplified by carefully siphoning off the medium above the cells in the culture bottle. The medium could be rapidly filtered. Then the yeast cells were poured from the culture bottle into the Buchner funnel and filtered as dry as possible. The yeast cells were then ready for autolysis, and the filtrate was discarded. A yield of about 400 grams wet weight was obtained.

The National Bureau of Standards (1) method for preparation of melibiase extract from brewer's yeast was followed to obtain an enzyme concentrate.

Drying Melibiase Concentrate

The ultrafiltered melibiase-invertase concentrate was placed in a 500 ml vacuum flask which was immersed in a water bath maintained at 35° C. Gentle magnetic stirring was used until the enzyme concentrate became too viscous for the stirrer to operate. Then the stirring bar was removed and the drying continued without agitation. About 12 hours were required for the final drying after which the dried melibiase-invertase is ready for use or storage. The yield was about 2 gms of dried concentrate.

Melibiase-invertase Activity Determinations

The activity of an enzyme is generally expressed in terms of the velocity of its specific reaction with a definite substrate.

The activity of melibiase and invertase were determined by observing the changes in optical rotation of melibiose and sucrose solutions through a period of time.

The hydrolysis velocity constant was calculated by the following formula:

$$k = -\frac{1}{t} \times \log \frac{P_i - P_f}{P_t - P_f}$$

Where k = the hydrolysis velocity constant
t = period of hydrolysis in minutes
P_i = initial polarization reading
P_f = final polarization reading
P_t = polarization reading at time t
k = 0.0040 for melibiase activity
k = 0.0225 for invertase activity

Another indication of melibiase-invertase activity can be obtained simply by taking polariscopic readings of a raffinose

solution at zero time and again after over-night hydrolysis. The ability of the enzymes to completely hydrolyze the standard amount of raffinose is a measure of activity which can be conveniently applied to sugar beet molasses analyses.

Raffinose hydrate, 1.35 gms, in water to make a volume of 50 ml was hydrolyzed overnight by 2 ml of a 5% solution of the dried melibiase-invertase concentrate. This is sufficient activity for the analysis of beet molasses wherein raffinose/100 solids is 6%.

Summary

The cultivation of *Saccharomyces carlsbergensis* in the laboratory for the production of melibiase-invertase has been described. An invertase-melibiase product satisfactory for the hydrolysis of raffinose was obtained from the laboratory grown yeast by following published procedures for the separation and purification of the enzyme.

Literature Cited

(1) United States Department of Commerce, National Bureau of Standards. 1942. Circular C 440 Polarimetry, Saccharimetry, and The Sugars. Pp. 147, 164.

(2) Reynolds, F. W. 1924. The rapid analysis of sugar, purification and concentration of enzyme solutions. Ind. and Engr. Chem. 16: 169, 172.