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Comparison of three different methods for trehalose determination in yeast extracts

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Trehalose is a disaccharide widely distributed in nature with great potential for application in different fields. Determination of trehalose has been carried out for many years using the anthrone colorimetric method, which may be subject to interferents. Alternatives to this problem seem to be the use of HPLC techniques or specific enzymatic assays. In the present work, a simple normal-HPLC procedure was applied to the analysis of different yeast extract samples and the results compared either to the conventional anthrone method or by an enzymatic assay based on the cleavage of trehalose by trehalase. Each method showed correlations over 0.97 with the others; however, only HPLC and the enzymatic method were statistically identical ($p < 0.05$). In addition, the HPLC method showed good linearity ($r = 0.995$) and recovery (98%), producing trehalose results in the range of 8.3–83 mg g^{-1} of cells. The results showed that in some cases the anthrone method may in fact produce inflated results although presenting consistency within the set of data. © 1997 Elsevier Science Ltd

INTRODUCTION

Trehalose is a glucose disaccharide widely distributed in nature and its presence in cells has many implications, particularly as a natural protector of membranes and proteins (Carpenter & Crowe, 1980; Crowe *et al.*, 1984). Consequently, its potential for preservation of biological systems including food is apparent.

Determination of trehalose has been carried out for many years by means of colorimetric methods, particularly the procedure based on the anthrone reaction (Brin, 1966). However, this method is subject to interferents which could also react with anthrone producing inflated results. In the last few years, some groups have described new approaches for trehalose determination by means of its specific hydrolysis by different trehalase preparations and subsequent determination of the glucose formed by the glucose oxidase-peroxidase reaction. (Schulze *et al.*, 1995; Petit & François, 1994; Van Aelst *et al.*, 1993; Araujo *et al.*, 1989; Tourinho dos Santos *et al.*, 1994). Chromatographic methods based on HPLC have also been applied either for trehalose confirmation (Vuorio *et al.*, 1993) or quantification using a Dionex system with an anion-exchange column and a pulsed amperometric detector (De Virgilio *et al.*, 1993). However, those HPLC methods have not been substantiated in terms of accuracy and precision. The Dionex system with a pulsed amperometric detector is a very sensitive technique but it is relatively expensive equipment and not easily available in most laboratories.

In the present work, results of 21 cell free extracts, obtained from seven different yeast strains grown on maltose and galactose, were compared for trehalose determination using the method of the anthrone reaction, one technique based on the specific cleavage of trehalose with the enzyme trehalase, and a chromatographic method based on high performance liquid chromatography (HPLC). Statistical analysis of the data and comments on the three methods are presented.

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MATERIALS AND METHODS

Yeast strains and growth conditions

The strains used in this study are listed in Table 1. Growth conditions consisted of 1.3% w/v yeast extract, 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, and 2% maltose or galactose. Cells were incubated at 28°C on a rotary shaker at 160 rpm in 500 ml flasks containing 100 ml of medium. Cell growth was followed by turbidity measurements at 570 nm.

Extraction

Trehalose was extracted from 10 mg of cells (dry wt) with 2 ml of 0.5 M trichloroacetic acid (Trevelyan & Harrison, 1956) and the extracts used for quantification by the anthrone method according to Brin (1966). When trehalose analysis was performed by using the trehalase method, the procedure described by Tourinho dos Santos *et al.* (1994) was followed. Trehalose extraction for HPLC analysis was carried out by means of boiling ethanol in a proportion of 40 mg of sample to 2 ml of ethanol. Ethanol was then evaporated and the residue dissolved in 1.5 ml of a solution of acetonitrile:water (1:1, v/v). This solution was then used directly for chromatography.

Trehalose determination

Anthrone method
Trehalose extracts were submitted to the reaction with anthrone and the colour formed measured at 620 nm, according to the procedure described by Brin (1966). Quantification was achieved using trehalose as external standard.

Trehalase method
Trehalose was determined by incubating the cell free extracts with a stable trehalase preparation (Tourinho dos Santos *et al.*, 1994). After incubation the glucose formed by trehalase activity was determined by the glucose oxidase peroxidase method (Zimmerman & Eaton, 1974).

HPLC method
The HPLC method used is based on a normal chromatography procedure, using a silica-amino column (250×4 mm i.d.) coupled to a guard-column (10×4 mm i.d.), both with the same stationary phase (Lichrospher-NH2, 250×4 mm i.d., particle size of 5 μm, Merck, Germany), acetonitrile-water (70:30, v/v) as mobile phase at 1 ml min^{-1} and a Knauer chromatograph coupled to a refractive index detector (Knauer, Model 64, Germany). The volume of injection which was 10 μl for both standard solutions and sample extracts, was achieved by means of a fixed loop injector Model 7125 (Rheodyne, USA). Identification and quantification were achieved by comparison of peak retention time and area of reference standards by means of an electronic integrator (Hewlett Packard Model 5987-A, USA). Linearity was checked with trehalose standards using five data points in the range 1–10 mg ml^{-1}, which was perfectly adequate to cover the range of interest. Precision of the method was assessed by calculation of the coefficient of variation of data obtained from six different extractions from the same sample (YSH 456) and recovery was estimated by addition of a known amount of standard to the sample before extraction. The conversion factor from mg ml^{-1} of solution to mg g^{-1} of cells was 57.1. The limit of detection of the proposed HPLC method was determined as the minimum amount of standard which could produce a peak:noise ratio of 2. Results were then compared with those obtained from the other two methods using a paired *t*-test from Statgraphics software.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
YSH 6.54.-1B	*MATa leu2-3/112 trp 1-92 ura3-52 MAL2-8^c GAL suc0*
YSH 456	*MATa tps2Δ:LEU2 leu2-3/112 trpl-92 ura3-52 MAL2-8^c GAL suc0*
YSH 488	*MATa ggsl/tpslΔ:TRPl tps2Δ:LEU2 leu2-3/112 trpl-92 ura3-52 MAL2-8^c GAL suc0*
JM 2763-14	*ura3 leu2 MAL1*
YSH 357	*ggsl/tpslΔ:LEU2 ura3 leu2 MAL1*
ENY.cat80-7A	*MATα leu2-3/112 ura3-52 CAT80 MAL2-8^c MAL3 SUC3*
YSH 368	*MATα ggsl/tpslΔ:LEU2 leu2-3/112 ura3-52 CAT80 MAL2-8^c MAL3 SUC3*

RESULTS AND DISCUSSION

Analysis of trehalose contents in yeast cell free extracts has been carried out for many years using the anthrone reaction (Brin, 1966). However, this is a non-specific method subject to interferents which can react with anthrone. Chromatographic methods based on HPLC seem to be the elected alternative to overcome this problem. However, the few HPLC reports for trehalose determination found in the literature were not substantiated in terms of precision and they use expensive and difficult to control detection systems (Vuorio *et al.*, 1993; De Virgilio *et al.*, 1993).

In the present work, an HPLC method is described which uses ordinary HPLC equipment (pump and refractive index detector) and shows adequate precision and simplicity for routine analysis of cell extracts. The method is based on a normal mode chromatography using a polar silica-NH_2 stationary phase and an acetonitrile:water solution as the mobile phase. These conditions proved to be perfectly adequate for the purpose of the work. Good linearity and recovery were obtained for the proposed method showing a correlation coefficient of 0.995 and 98% of recovery for trehalose. The method also showed very good precision as assessed by determination of the coefficient of variation (5.1%). The described HPLC procedure was then compared with the anthrone and trehalase methods and results are shown in Table 2. Each method presented very good correlation with the others (HPLC× anthrone = 0.97; HPLC× trehalase = 0.98; anthrone × trehalase = 0.97; $p < 0.05$). However, application of the paired *t*-test showed that no significant difference ($p < 0.05$) was only found between HPLC and trehalase. This was due to the fact

Table 2. Trehalose determination in yeast strains obtained by three different methods

Strain	Carbon source \ cell concentration (mg ml^{-1})	Trehalose determination by: HPLC	Anthrone	Trehalase
JM 2763-14	Galactose\1.6	29.6	38.0	35.0
JM 2763-14	Maltose\2.0	69.6	71.6	64.0
JM 2763-14	Maltose\1.8	99.1	100.7	91.0
YSH 654-1B	Maltose\1.7	69.6	77.4	62.5
YSH 456	Maltose\1.3	15.0	10.7	18.0
YSH 456	Galactose\2.9	16.0	24.6	16.0
YSH 488	Galactose\1.2	11.0	19.0	12.0
YSH 488	Galactose\2.9	8.3	18.5	12.0
YSH 488	Galactose\1.8	11.7	17.2	13.6
YSH 488	Maltose\1.7	18.0	45.5	24.0
YSH 488	Maltose\2.0	32.6	41.0	28.8
ENY.cat 80-7A	Galactose\2.6	67.0	70.3	76.0
ENY.cat 80-7A	Galactose\3.3	58.6	72.3	64.0
ENY.cat 80-7A	Maltose\1.2	62.4	66.0	57.0
ENY.cat 80-7A	Maltose\2.0	83.0	96.4	88.0
ENY.cat 80-7A	Maltose\2.7	53.0	72.3	64.0
YSH 368	Galactose\1.8	14.8	15.7	15.0
YSH 368	Galactose\2.3	11.7	12.9	9.5
YSH 368	Maltose\1.4	10.0	22.9	9.5
YSH 368	Maltose\1.9	10.0	26.6	12.3
YSH 357	Maltose\1.8	17.7	27.4	16.0

Results are average of duplicate determinations expressed in mg g^{-1} of cells.

that all methods have good precision but the anthrone method tends to produce inflated results, probably due to interferents which do not affect the HPLC and trehalase methods.

The detector used for HPLC analysis was the refractive index detector which is known to have low sensitivity. Another limitation of using this detector is the impossibility of gradient elution utilisation. However, these two limiting conditions did not negatively affect the proposed HPLC method since a clean extract was obtained facilitating trehalose separation from other interferents and, in addition, the extracts from yeast cultures could be easily obtained in the concentration range adequate for detection with the refractive index monitor. The limit of detection of the method was next to 0.5 μg on column which is perfectly adequate for the proposed application. Identification of trehalose was confirmed by comparison of retention time of reference standard, by coelution of sample with standard, by running both standard and sample at different chromatographic conditions and also by agreement with the results obtained by the specific trehalase method. Chromatograms of standard and of a yeast extract sample are presented in Fig. 1. Few small peaks, well separated from trehalose, can be seen in the sample chromatogram but major interferents which could be responsible for inflated results in the anthrone method are probably co-eluting with the solvent front where pentoses and other less polar compounds are expected to appear.

In conclusion, the comparison of the three methods showed that the proposed HPLC procedure for trehalose analysis is in perfect agreement with the results obtained by the specific enzyme assay and is, therefore, very useful in genetic and biochemical studies involving trehalose synthesis and degradation, in trehalose determination in biological samples as well as in the investigation of more productive yeast strains for future technological applications of trehalose. Although the anthrone method produces inflated results for trehalose contents in biological samples, this method is useful in

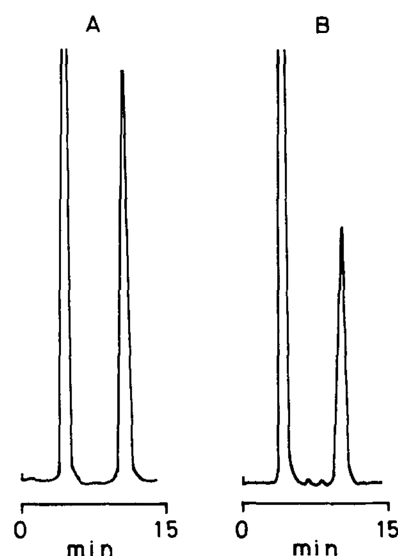

Fig. 1. Chromatograms of trehalose standard (A) and yeast extract sample (B). Chromatography was carried out with a Lichrospher-NH2, 250×4 mm i.d. column, acetonitrile-water (70:30, v/v) as mobile phase at 1.0 ml min^{-1} and refractive index detection. Concentrated (2×) extract of sample ENY grown with 2 mg ml^{-1} of maltose was used.

routine screening procedures since it is the cheapest method available for this purpose and the values obtained, although inflated in some instances, do not exclude the presence of trehalose in cell free extracts.

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