Screening of Extractant for Determining Active Sludge Trehalose in Sewage Treatment

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This study investigated and proposed the use of trehalose extraction and a detection method for the determining of active sludge trehalose in sewage treatment. Seven extractants (trichloroacetic acid, ethanol, methanol, acetone, pure water, formaldehyde and trichloromethane) were used separately to extract the active sludge trehalose, and their trehalose contents were determined. The results shown in standard curves plotted for all seven extractants demonstrated good linearity, and the regression coefficients varied insignificantly. Using trichloroacetic acid, active sludge trehalose was extracted within a period of only 40 min at 40 centigrade. In view of that, trichloroacetic acid proved to be as the most efficient extractants in extracting trehalose from active sludge. Its extraction rate was 4 to 11-times faster than that of other extractants for the same amount of active sludge. From our results, trichloroacetic acid was substantiated as the optimal extractant for determining active sludge trehalose.

Keywords Active sludge, trehalose, extractant

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Introduction

Trehalose is a non-reductive disaccharide containing two glucose molecules connected by the α - α -1-1-glucosidic bond (molecular formula, $C_{12}H_{22}O_{11} \cdot 2H_2O$; molecular weight, 378.33). It is chemically stable, non-toxic, cannot be caramelized and exists in the form of a white crystal. Trehalose demonstrates strikingly protective effects for organisms by acting as an important metabolite in organisms ability to respond to environmental stress.¹⁻⁶ This anti-stress ability is associated with the content of trehalose present in organisms challenged by an adverse environment. An enhanced synthesis of trehalose can counter potential damages caused by adverse factors. The determination techniques for trehalose in living organisms have been extensively applied in the food, medicine and cosmetics industries. Nevertheless, environmental engineering still represents an unexplored, but promising, field of application for trehalose.

The primary functions of trehalose known in biology are non-specific protective and anti-radiation effects for organisms.⁷⁻¹¹ Active sludge, as a product at sewage treatment plants, in many engineering situations still demonstrates digestibility, even below temperatures as low as -30 to -40°C .¹²⁻¹⁵ Microorganisms with a large amount of trehalose are protected from temperature variations and harsh environments (e.g. drought, high-salt and extreme temperature).¹⁶⁻¹⁹ By establishing an appropriate approach for the quantitative determination of active sludge

trehalose in biological sewage treatment, we will be able to identify microorganisms that are resistant to high salt stress, cold and toxicity (or are suitable) to be used in the research and application of the trehalose synthase gene. Considerable variations in the load, ambient temperature and pH value will greatly influence the trehalose content in sludge containing large quantities of microorganisms that digest sewage. The trehalose content is a sensitive indicator for detecting environmental stimuli. Exogenous trehalose can be used to enhance the stress response ability of active sludge.

The most common method for trehalose quantification is anthrone colourimetric, based on the principle that in the presence of sulfuric acid, the dehydration of trehalose can result in the formation of furfural or hydroxymethylfurfural. The anthrone then reacts with furfural or hydroxymethylfurfural to form a blue-green complex, measured by spectrophotometry. The extractant used largely determines the accuracy of trehalose detection. Many studies have been carried out in relation to selecting the extractant, but the results vary from one experiment to another. Espinosaa *et al.* were the first to obtain trehalose using pure water from a variety of yeasts in 1950.²⁰ Plourde-Owobi *et al.* satisfactorily extracted trehalose using trichloroacetic acid,²¹ which was, however, a strong protein denaturant, but this method were not suitable for mass production. Existing studies by Chinese scholars showed the extraction of trehalose using by pure water, ethanol, trichloroacetic acid, acetone, methanol, formaldehyde and trichloromethane. Zhang *et al.* crushed yeast cells by microwave-assisted pretreatment and extracted trehalose using pure water,²² ethanol and trichloroacetic acid, respectively. The extraction performance was obviously higher with a microwave-

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Table 1 Character of the extraction solvents

Extraction solvent	Density/ g mL ⁻¹	Boiling point/ centigrade	Melting point/ centigrade	Solubility/ g
Trichloroacetic acid	1.63	197.5	57.5	Dissolved
Ethanol	0.7893	78.4	-117.3	Dissolved
Methanol	0.791	64.5 - 64.7	-98	Dissolved
Acetone	0.898	56.5	-94.6	Dissolved
Formaldehyde	1.085	-19.5	-118	Dissolved
Pure water	1.000	100	—	Dissolved
Trichloromethane	1.4916	61.2	-63.5	1.0

assisted pretreatment compared to that without it.

Currently, trehalose extraction and detection are mostly involved in the food industry, molecular biology, agriculture, and the pharmaceutical and cosmetics industries as an analysis procedure, but not yet used in detecting sewage by means of active sludge. In this work, we proposed a conventional procedure for extraction and detection of trehalose quantification for active sludge trehalose in sewage treatment. Seven extractants, namely trichloroacetic acid (TCA), ethanol, methanol, acetone, formaldehyde, pure water and trichloromethane, were used, respectively, to extract active sludge trehalose. The optimal extractant was identified from the standard curves plotted from the results. This work aims to propose a unified method for determining the content of active sludge trehalose. Table 1 gives the properties of the seven extractants.

Material and Methods

Experimental sludge

Aerobic sludge was collected from an already operating sequencing batch reactor in our laboratory.

Determination of the content of active sludge trehalose

After 0.8 mL of an active sludge sample was measured and transferred into a 10-mL centrifuge tube and centrifuged at 4500 r/min for 5 min, the tube was washed with distilled water and centrifuged again under the same condition. This procedure was repeated three times. Then, 6 mL of 0.4 mol/L trichloroacetic acid solution was added after the supernatant was removed. The sludge sample was treated ultrasonically at 25 KHz for 40 min, and was later centrifuged at 4500 r/min for 8 min. The obtained supernatant was the trehalose extract. Then 10 mL of anthrone and 5 mL of the trehalose extract were mixed sequentially in a 50 mL fermentation tube, which was cooled immediately in a cold water bath. Next, the tube was taken out and placed in a boiling water bath for 6 min. Afterwards, the tube was cooled to room temperature. The absorbance was measured at 610 nm using a 1-cm cuvette. The sludge sample was treated ultrasonically, centrifuged under the above-mentioned conditions and dried at (105 ± 1) centigrade for 1 h, and weighed afterwards. The content of trehalose in the sludge bacteria was calculated from the absorbance and the weight of the dried sludge.

The content of trehalose, C , in the sludge bacteria is given by

$$C = \frac{KOD_{610}}{W}, \quad (1)$$

where C is the content of trehalose (mg) of the active sludge in the mixture (mg L⁻¹/mgMLSS); K is the slope of the standard curve; W is the weight of the dried sludge (mg); OD_{610} is the absorbance of the trehalose-anthrone mixture at 610 nm.

Note: Equal amounts and concentrations of all seven extractants were taken, 6 mL and 0.4 mol/L, respectively, to ensure the accuracy and precision compared to the trehalose extracts.

Results and Discussion

Comparison of standard curves

Plotting of standard curves. For each of the extractants, the trehalose extract from the sludge samples was quantified using the standard curves. The standard curves were characterized by two parameters: 1) The extraction constant (for the standard curve $y = k_e x$, where k_e is the extraction constant, representing the absorbance of trehalose dissolved per unit volume of extractant). The larger was the extraction constant, the stronger was the ability of the extractant to extract trehalose; 2) Regression coefficient. The higher was the regression coefficient, the higher was the linearity of the standard curve plotted using this extractant.

Since trichloroacetic acid, ethanol, methanol, acetone, formaldehyde and pure water were miscible with water, there was no extractant-water layer stratification when trehalose was extracted. Instead, the trehalose was evenly distributed in the mixture of water and the extractant. The volume ratio between the extractant and water was 1:1; thus, the trehalose concentration in the extractant was reduced to half of its original concentration. In this case, the measured absorbance should be half of its corresponding pure extractants. The absorbance values plotted on the standard curves were doubled in order to consider this problem fully. For trichloromethane, which was slightly soluble in water, the measured value of solubility was also adjusted. Normally, 1 g (or 0.67 mL) of trichloromethane dissolves in 1 mL of water, but when 6 mL of trichloromethane was added to 6 mL of water in this experiment, only 4.02 mL dissolved in water. Therefore, the measured absorbance was multiplied by a factor of 0.33 (the volumes of the extractants obtained were all 7 mL).

Because trichloroacetic acid, ethanol, methanol, acetone, formaldehyde and pure water are soluble with water, liquid-liquid stratification did not occur when the two extractants were used to make standard curves. Trehalose was evenly distributed in the mixed solution of water and the extractant. Since the volume ratios of trichloroacetic acid, ethanol, methanol, acetone, formaldehyde and pure water to water are all equal (thus 1), the trehalose concentration in the extractant was diluted twice, so the absorbance measured in this case was 1/2 of trehalose in a single trichloroacetic acid, ethanol, methanol, acetone, formaldehyde and pure aqueous solution. In order to reflect the problem, the values on the standard curves of trichloroacetic acid, ethanol, methanol, acetone, formaldehyde and pure water (as shown in Fig. 2) were adjusted to twice the initial values. The measured values of trichloromethane were also modified according to the apparent solubility. For example, in the case of 1 mL of water dissolving 1 g or 0.67 mL of trichloromethane, only 4.02 mL of trichloromethane dissolved in 6 mL of water solution when 6 mL trichloromethane was added. The measured absorbance value was multiplied by 0.33 because the volume of the extractant on the standard curve was 7 mL.

Figure 1 shows the standard curves fitted for the seven extractants. All values plotted on the curves were averages

obtained from several measurements, as in the other experiments described in this article. The seven standard curves demonstrated good linearity, and the regression coefficients did not vary significantly. In the decreasing order of the extraction constant, the extractants were ranked: trichloroacetic acid > acetone > pure water > trichloromethane > methanol > ethanol > formaldehyde. *UV-Vis spectral patterns.* UV-Vis spectroscopy was used to detect the trehalose extracts from the different extractants. The extract with the closest UV-Vis spectral characteristics to that of the trehalose standard solution was found by a power fit. The larger was the power fit coefficient, K_m^n , the closer was the concentration of trehalose in the extract to that of the standard solution. The optimal absorption wavelength was fitted based on the trough values on the UV spectra. The results of a full-

Fig. 1 Comparison of type curves for seven tested extraction solvents.

range UV-Vis scan for trehalose extracts using trichloroacetic acid, ethanol, 50% ethanol, methanol, acetone, pure water, 37% formaldehyde and trichloromethane, are shown in Fig. 2.

After detecting and extracting the trehalose content of the sludge, the amount of trehalose in the sludge sample was determined and quantified by the standard curve. Therefore, the function of the standard curve cannot be ignored. In general, the characteristics of the standard curve produced by the extractant can be reflected by the following two parameters; 1) The extraction constant (in the standard curve $y = k_e x$, k_e is defined as the extraction constant. This represents the absorbance of trehalose dissolved by a unit volume of the extractant). A large extraction constant suggests the ability of this extractant to dissolve trehalose. 2) The regression coefficient. A large regression coefficients showed that the seven extractants had good performance in making standard curves. Standard curves were constructed as done previously.⁸

The power fit was performed using Origin software. The decreasing order of the fit coefficient was $K_m^n_{\text{Trichloroacetic acid}} = 0.984 > K_m^n_{\text{Methanol}} = 0.827 > K_m^n_{\text{Pure water}} = 0.628 > K_m^n_{\text{Acetone}} = 0.535 > K_m^n_{\text{Trichloromethane}} = 0.478 > K_m^n_{\text{Ethanol}} = 0.425 > K_m^n_{\text{Formaldehyde}} = 0.301$. The optimal wavelength fitted was 610 nm. Among the seven extracts, the UV-Vis spectrum of trehalose extract using trichloroacetic acid was the closest to that of the trehalose standard solution. This suggested that the extract using trichloroacetic acid mainly contained trehalose.

Comparison of the amount of trehalose extracted from sludge

Extraction time under 40 centigrade. The extraction time is one of the major performance indicators of the extractant. It refers to the time required to extract the maximum amount of trehalose from the sludge for a certain extractant, and reflects the extraction rate. A reagent can extract the highest amount of trehalose, but considered to be impractical if it takes excessive time to finish the extraction. The seven extractants were compared in terms of their extraction time, and the optimal

Fig. 2 Comparison of the ultraviolet spectrum for the tested extraction solvents.

Fig. 3 Trehalose extraction as a function of time for several kinds of extraction solvents at 40 centigrade.

extraction time in subsequent experiments. Because the trehalose is usually synthesized and stored in bacterial cells, it cannot be extracted and measured except by destroying the bacterial cell wall. Therefore, the sludge samples were first pre-treated at 40°C at 25 kHz using an ultrasonic cell disruption apparatus, as described in the previous section. When using trichloromethane as an extractant, the sludge agglomerated and adhered to the tube wall once it was exposed to the extractant. This implies that trehalose cannot be extracted using trichloromethane under 40°C. Figure 3 shows the results of trehalose extraction using the other six reagents.

It can be seen from Fig. 3 that the trehalose amount extracted from the sludge samples reached its maximum at 40 min when using trichloroacetic acid. The extraction time was at least 50 min for the other extractants. Based on the amount of trehalose extracted, extraction time and the corresponding standard curves (Fig. 1) the extraction rate for each extractant was calculated. Under a temperature of 40°C and equal amounts of sludge, the extraction rates of trichloroacetic acid, formaldehyde, pure water, acetone, methanol and ethanol were 8.65, 2.37, 2.03, 1.91, 1.03, and 0.71 $\mu\text{g} \cdot (\text{mL} \cdot \text{min})^{-1}$, respectively. The extraction rate of trichloroacetic acid was approximately 4-times more than that of formaldehyde, pure water and acetone, 8-times that of methanol, and 11-times that of ethanol.

The experimental conditions were basically consistent in most of the previous studies, except for the extraction time, which was specifically determined by the amount sludge used. Trichloroacetic acid was investigated at 40°C in another experiment, as described in this article (Fig. 4), to determine whether the sludge amount can be affected by the actual extraction time. The results showed that trichloroacetic acid needed only 40 min to extract the maximum amount of trehalose from several volumes of sludge. Therefore, when using trichloroacetic acid, the sludge amount has no obvious influence on the extraction times. The larger is the sludge amount, the higher is the trehalose amount contained in the sludge; consequently, there is a higher probability for trehalose exposure to the extractant, and accordingly, the acceleration of the extraction rate. The optimal extraction time for trichloroacetic acid was 40 min.

The ability to extract trehalose from sludge under 40°C. This experiment compared the abilities of the six reagents to extract trehalose from sludge samples. The extraction time for each extractant was determined according to the results shown in

Fig. 4 Trehalose extraction as a function of time by acetone under different sludge amounts at 40 centigrade.

Fig. 5 Comparison of trehalose extraction from activated sludge by several kinds of extraction solvents at 40 centigrade.

Fig. 2 (trichloroacetic acid, 40 min; formaldehyde, 50 min; pure water, 60 min; acetone, 60 min; methanol, 70 min; and ethanol, 90 min). The extraction results are given in Fig. 5.

It can be seen from Fig. 5 that trichloroacetic acid possessed the highest ability to extract trehalose from the sludge sample. Its absorbance was also the largest, followed by formaldehyde. A comparison with Fig. 1 reveals the inconsistency between the fitted standard curves and the actual abilities of the extractants to extract trehalose from the sludge. For example, acetone had a high extraction constant and a high ability to dissolve trehalose, as indicated by the standard curve. However, according to Fig. 5, its ability to extract trehalose from the sludge (solid composition were not treated ultrasonically treated) was poor. This is because the extraction did not correspond to liquid-liquid extraction in this test (Fig. 5), but rather to the liquid-solid extraction. The limiting factor was the ability of the extractant to destroy the bacterial cell wall and cell membrane before extraction could occur. During the liquid-liquid extraction performed for fitting the standard curves, the limiting factor was the solubility of the trehalose in the extractant. The content of trehalose in the sludge was determined using the standard curve and the measured absorbance of the resulting mixture for each extractant. As Fig. 5 shows, the amount of trehalose extracted using the six reagents were ranked in the same order as the measured absorbance. Trichloroacetic acid extracted the largest

amount of trehalose. The trehalose contents (calculated using the standard curves) were proportional to the abilities of all six reagents in extracting trehalose from the sludge. Such consistency was confirmed by the small difference between the plotted standard curves. Finally, trichloroacetic acid was determined as the optimal extractant, since it showed the shortest extraction time and the highest extraction ability.

Conclusions

The standard curves fitted by using seven reagents to extract trehalose from the sludge were basically consistent. UV-Vis spectroscopy was performed for all trehalose extracts. A comparison of the spectra revealed that the extract obtained using trichloroacetic acid contained the closest amount of trehalose to that of the standard solution. Trichloroacetic acid, among all seven reagents, had the shortest extraction time, the highest extraction rate and the strongest extraction ability (at temperatures under 40°C). Its extraction constant and the regression coefficient were also high. Therefore, trichloroacetic acid is the optimal extractant for the determination of active sludge trehalose.

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