Analytical Methods

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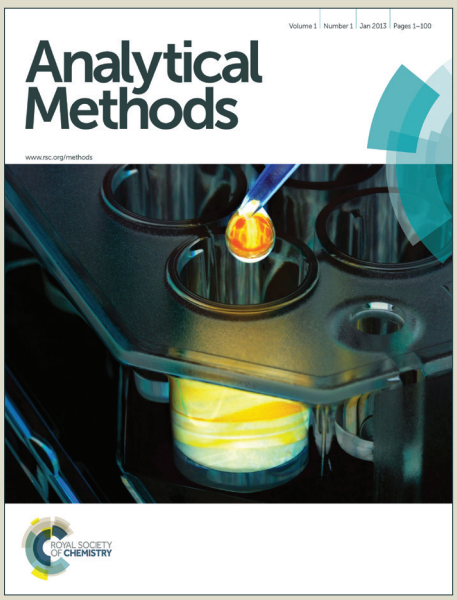

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A Rapid method to assess Reactive Oxygen Species in yeast using H_2DCF-DA

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A protocol to efficiently assess Reactive Oxygen Species (ROS) levels in yeast cells using H_2DCF-DA is described here. This method employs lithium acetate to permeate the cell wall, and thus, augments the release of the fluorescent product, dichlorofluorescein from the cells. This protocol obviates the need for both physical and enzymatic lysis methods that are arduous and time consuming. This method is simple, less time consuming and reproducible, specially while dealing with a large sample size. The lithium acetate method gave significantly reproducible and linear results (P<0.0001), as compared with direct measurement (P=0.0005), sonication (P=0.1466) and bead beating (P=0.0028).
Key words: H_2DCF-DA, Reactive Oxygen Species, Yeast

1. Introduction

The H_2DCF-DA assay is a technique that is widely used to profile the oxidative status of living cells. It was initially developed by Brandt *et al* in 1965 [1]. The assay is based on cellular esterase action on H_2DCF-DA to cleave acetate groups on the moiety, releasing an intermediate H_2DCF which reacts with ROS in its vicinity to form a fluorescent product, 2',7'-dichlorofluorescein (DCF) [2]. H_2DCF is converted to the fluorescent DCF moiety by reaction with hydroxyl radicals ($^{\bullet}OH$), peroxidase compounds I and II arising from catalysis of H_2O_2, $^{\bullet}NO_2^-$ generated from the myeloperoxidase /H_2O_2/ NO_2^- system, hypochlorous acid (HOCl), and reactive species produced from peroxynitrite ($ONOO^-$/ONOOH) decomposition. $^{\bullet}OH$ or carbonate anion radicals ($CO_3^{\bullet-}$) are also known to be formed during Peroxynitrite decomposition [3]. Based on this, several methods have been developed for quantitative and qualitative assessment of ROS production. Since ROS are short-lived, transient species and are difficult to estimate directly *in vivo*, only a few methods that measure ROS have been widely accepted [2]. The H_2DCF-DA method gives an indirect measurement of the ROS content in the cell, and therefore, any interference from procedures employed in sample processing would yield high error values and could lead to nuances in data interpretation [2]. Several researchers use H_2DCF-DA to estimate cellular ROS and hence, there exists a need for more reliable methods. Despite the advent of advanced techniques such as flow cytometry to analyse fluorescence in intact cells, determination of H_2DCF-DA oxidation using a fluorescence spectrophotometer is commonplace in several laboratories worldwide owing to its cost-effectiveness. The usual method for ROS assessment in yeast involves the incubation of cells for 30 minutes with the dye, following which, fluorescence intensity is measured either directly (for intact cells), or using a cellular lysate. It is known that shear stress and sonication can increase ROS production *in vitro* [4–7] and this could interfere with the results. To circumvent this problem, we developed a lithium acetate-SDS based method to reduce the influence of externally generated ROS that could interfere with the results. In this paper, the various methods of ROS measurement by DCF fluorescence have been compared to the Lithium Acetate-SDS method. The Lithium Acetate-SDS method seems efficient, less time consuming and gives consistent results for a large sample size.

2. Materials and Methods

The different methods used in this work were assessed in *Saccharomyces cerevisiae* MTCC 170. Yeast grown overnight was inoculated into fresh YEPD medium at room temperature, until the cells reached the exponential phase. Unless otherwise mentioned, ~1.5×10^7 CFU/ml (OD_{660} = 0.9) cells were taken per sample for each experiment. H_2DCF-DA was procured from Sigma Aldrich, India. Fluorescence assays were performed using a Jasco spectrofluorimeter FP8600 (Tokyo, Japan) interfaced with a computer, using a 1cm path length quartz cuvette. Cells were taken in ascending order and the linearity of H_2DCF-DA fluorescence was assessed using one-way ANOVA followed by post-hoc linear trend analysis between columns (P values < 0.05 were considered significant). The cell number was kept constant to assess the efficiency of the different protocols used. Yeast cultures were harvested and centrifuged at 6000g for 10 minutes and the pellet was washed twice in phosphate buffer (0.1M, pH 7.4). Cells were incubated for 30 minutes with H_2DCF-DA (10µM) in the same buffer in the dark and then centrifuged. The washed pellet was taken for further analysis for all the methods. The excitation and emission wavelengths used for H_2DCF-DA were 504nm and 524nm, respectively.

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2.1 Direct fluorescence of the cells

Yeast cells were centrifuged and the pellet was incubated in H_2DCF-DA (10μM) for 30 minutes and then centrifuged at 6000g for 10 minutes. The pellet was then resuspended in phosphate buffer and fluorescence of the cells was measured [8].

2.2 Lysis by glass beads

The method was adapted from Cabiscol *et al* [9]. After H_2DCF-DA incubation, the cells were resuspended in distilled water and disrupted by vortexing with glass beads (Sigma, 400-625 μM). The resulting extract was assessed fluorimetrically.

2.3 Lysis by Sonication

The pellet obtained after H_2DCF-DA incubation was suspended in phosphate buffer, and then sonicated for 5 one-minute intervals on full power (25KHz, 150W) and maintained on ice for one minute in between the intervals. The entire cell lysate was then taken for fluorescence measurements.

2.4 Enzymatic Lysis of Yeast

Enzymatic lysis was carried out as described by Martins *et al* [10]. The cells were gently washed in a solution containing sorbitol (1.2M), EDTA (50mM) and mercaptoethanol (2%). After centrifugation at 6000g for 10 minutes, the pellet was resuspended and gently agitated for 30 minutes in same buffer with lyticase (25 U/ml) to generate spheroplasts. The spheroplasts were then incubated in H_2DCF-DA (10μM) for 30 minutes. The cells were centrifuged and the pellet was resuspended in phosphate buffer and then mildly sonicated. The resulting lysate was used immediately for fluorescence measurements.

2.5 Lithium Acetate-SDS method

After H_2DCF-DA addition, the cells were resuspended in 2M lithium acetate (LiAc) and gently agitated for 2 minutes, and then centrifuged immediately. The resulting pellet was suspended in 0.01% SDS with one drop of chloroform, for 2 minutes with vigorous agitation. This facilitated exclusion of dye from the cells. Agitation done with cells suspended only in distilled water yielded similar results. The samples were centrifuged at 6000g for 5 minutes and the supernatant was used for fluorescence measurement.

3. Results and discussion

Method	Number of cells	N	Mean Fluorescence	Standard Deviation
Cell Suspension	1.5×10^7	8	94.08	37.47
Lithium Acetate-SDS	1.5×10^7	8	233.96	24.19
Sonication	1.5×10^7	8	774.00	203.24
Enzymatic Lysis	1.5×10^7	8	1487.25	305.15
Glass Bead	1.5×10^7	8	1578.00	244.41

Table 1. Standard deviations from the mean fluorescence of 8 (N) samples containing equal number of yeast cells

3.1 Using whole cells to assess fluorescence could generate variations across a large sample size

Assessing the samples directly without sonication or by mild sonication gave slightly altered signals. Yeast being a suspension, mostly tend to settle over time and while estimating ROS for a large sample size, errors across samples could occur. Although a linear trend was observed (P=0.0005), due to variations in processing time from sample to sample, there could be ROS generation in cells. Therefore, values obtained for fluorescence signals generated by whole cells were not consistent (S.D=34.47), as seen in Table 1.

3.2 Lysis using glass beads

Lysis by glass beads is efficient in facilitating protein extraction[11], but sometimes cells fail to lyse evenly, especially when they are beyond the exponential phase of growth. Also, cell lysis is a time-consuming process, during which ROS generation can vary from sample to sample. Judging from the standard deviation in the data (S.D=244.41) (Table 1), for a large sample size, one could reconsider the use of this method to save time and increase efficiency. Lysis by glass beads gave linear results with varying cell number (P=0.0028); but as we have observed, for a large sample size (Figure 1A), bead beating could yield an exaggerated value *in vivo*, by escalating ROS production and this could be due to the time factor involved and also due to shear stress[7].

3.3 High standard deviation from sonication and enzymatic lysis

Cellular lysis by sonication gave results that showed a high standard deviation (S.D=203.24), which could probably be attributed to inconsistent lysis and ROS production during the method itself (Figure1A). Also, heat generation due to sonication probably led to an altered ROS signal. This is highly undesirable, since ROS generated in an experiment by the method could give rise to errors in data interpretation. Enzymatic generation of spheroplasts prior to lysing the cells did not differ very much from sonication, in yielding a high standard deviation (S.D=305.15). Enzymatic lysis of the cell to obtain spheroplasts, followed by sonication is an efficient method to lyse cells. However, the fact that reagents like mercaptoethanol are added to the lysis buffer cocktail invalidates the usefulness of the protocol because of high interference from these chemical agents. Therefore, using this method before or after the treatment of the dye is not recommended. Another major factor that impedes the entire purpose of assessing ROS is time. The time variation across samples during sonication or enzymatic lysis also could contribute to undesirable ROS generation; this is because the level of ROS generation is inexplicable across a given time frame. There is no linear trend observed between the number of cells used and the ROS generated (Figure 1C(iii)). Therefore, when dealing with a large sample size, one must reconsider the usage of sonication or enzymatic lysis, which could yield erroneous results and a fallacious picture of the *in vivo* redox status of the cell.

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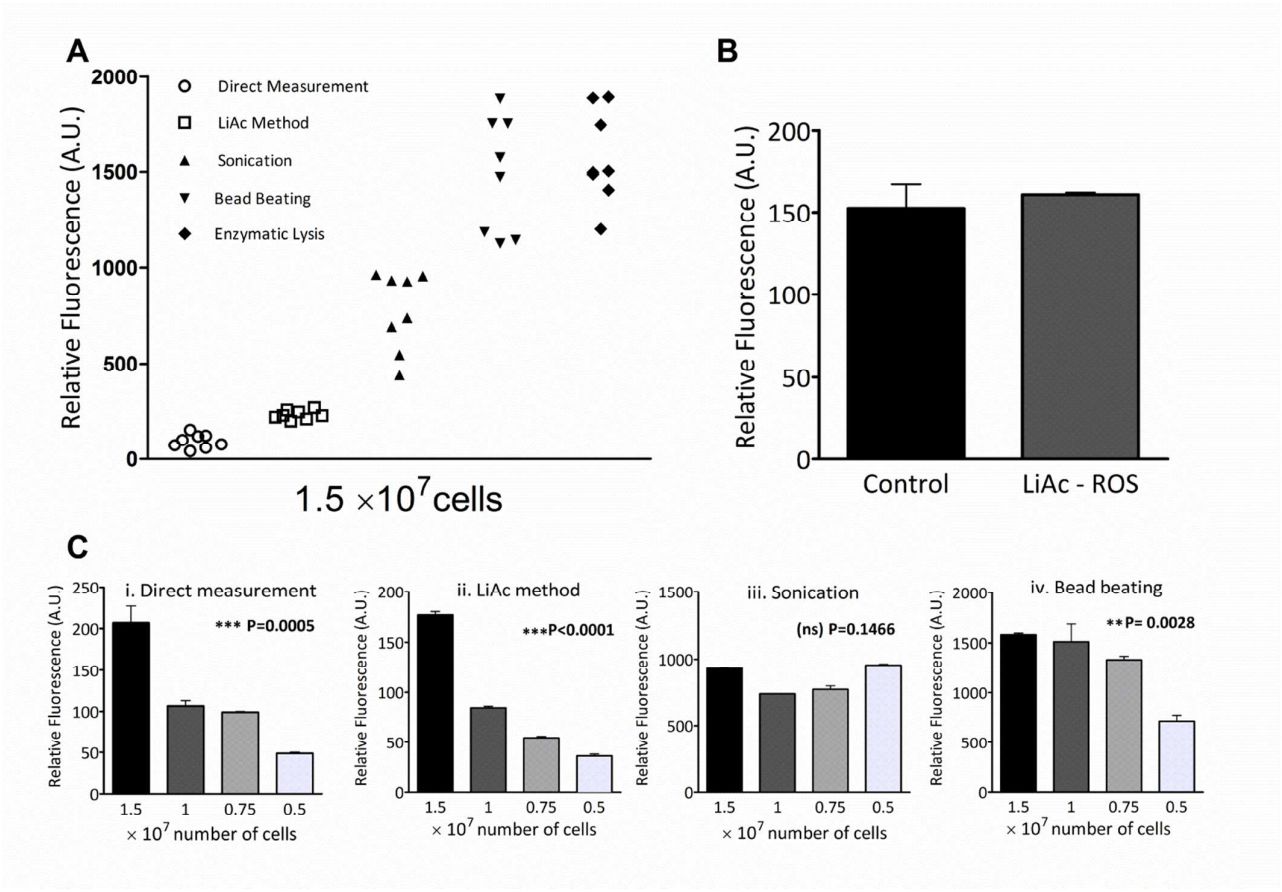

Figure 1. (A) Variation in ROS levels generated by different treatments. The number of yeast cells was 1.5 ×10^7 per treatment **(B)** Treatment of 0.5 ×10^7 yeast cells with Lithium acetate-SDS **(C)** Linearity of the methods employed: i. Direct fluorescence of a yeast cell suspension ii. Fluorescence of the extract after Lithium acetate procedure iii. Fluorescence of lysate after sonication iv. Fluorescence of lysate after glass bead beating. All samples were diluted sequentially from 1.5 to 0.5 ×10^7 cells the from stock culture

3.4 Consistency of the Lithium Acetate method

The extraction method of Brennan and Schiestl [12] gave an erratic ROS signal due to the prolonged incubation time with SDS. However, treating the samples with lithium acetate and SDS for a little over 2 minutes did not generate much ROS (Figure 1B). Also, the lithium acetate method gave consistent results (S.D=24.19) (Table 1). The extraction of the dye from the cells into the supernatant by agitation was uniform across all samples (Figure 1A), and thus, a large number of samples could be analysed within a limited time frame. This method circumvents the undue increase in ROS generation during prolonged incubation periods. Also, this method is sensitive for a small number of cells and give readings that can be highly correlated (P<0.0001, Figure 1C(ii)). Therefore, for a large sample size, this method could be employed as an alternative to probe the oxidative status of yeast cells by avoiding superfluous ROS generation in the samples.

4. Conclusions

ROS estimation using the H_2DCF-DA method is error-prone due to several possible interferences [2]. Yet, it is one of the most widely used methods. We suggest that sonication and enzymatic lysis be avoided in experiments that estimate ROS because the methods by themselves are known to generate ROS [4–6]. Bead beating and direct fluorescence are indeed efficient methods in ROS estimation but are to be used with caution in experiments with a large sample size. The Lithium Acetate-SDS method is seen to be both relatively efficient and consistent and could be employed in estimating ROS for a large sample size. The Lithium acetate-SDS method does not contribute to an erratic increase in ROS, because this method can be simultaneously performed for all the samples in a given experiment. This method gives relatively error free results, without

yielding additional ROS generation and gave values similar to those for samples directly assessed for their fluorescence.

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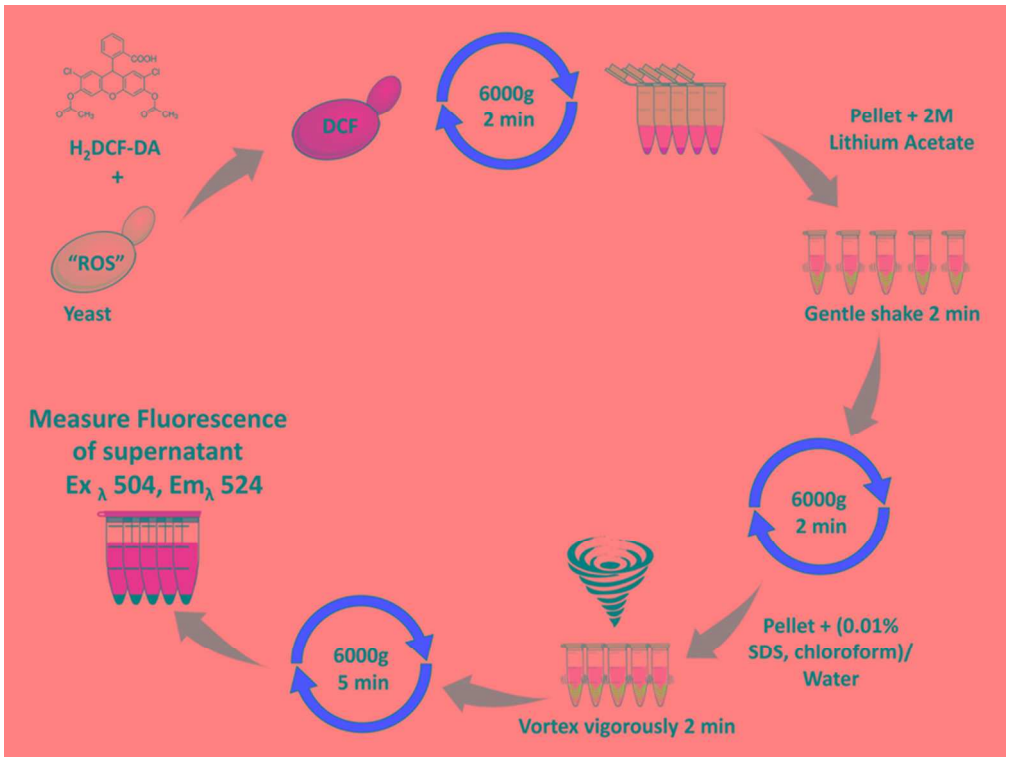

A rapid method to extract DCF from yeast to measure its oxidative status by a fluorescent spectrophotometer
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