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Rapid Fluorescence Assay for Polyphosphate in Yeast Extracts Using JC-D7

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ABSTRACT

Polyphosphate (polyP) is an intriguing molecule that is found in almost any organism, covering a multitude of cellular functions. In industry, polyP is used due to its unique physiochemical properties, including pH buffering, water binding, and bacteriostatic activities. Despite the importance of polyP, its analytics is still challenging, with the gold standard being ^{31}P NMR. Here, we present a simple staining method using the fluorescent dye JC-D7 for the semi-quantitative polyP evaluation in yeast extracts. Notably, fluorescence response was affected by polyP concentration and polymer chain length in the 0.5–500 μg/mL polyP concentration range. Hence, for polyP samples of unknown chain compositions, JC-D7 cannot be used for absolute quantification. Fluorescence of JC-D7 was unaffected by inorganic phosphate up to 50 mM. Trace elements ($FeSO_4 > CuSO_4 > CoCl_2 > ZnSO_4$) and toxic mineral salts ($PbNO_3$ and $HgCl_2$) diminished polyP–induced JC-D7 fluorescence, affecting its applicability to samples containing polyP–metal complexes. The fluorescence was only marginally affected by other parameters, such as pH and temperature. After validation, this simple assay was used to elucidate the degree of polyP production by yeast strains carrying gene deletions in (poly)phosphate homeostasis. The results suggest that staining with JC-D7 provides a robust and sensitive method for detecting polyP in yeast extracts and likely in extracts of other microbes. The simplicity of the assay enables high-throughput screening of microbes to fully elucidate and potentially enhance biotechnological polyP production, ultimately contributing to a sustainable phosphorus utilization.

1 | Introduction

Inorganic polyphosphate (polyP) is the linear polymer of phosphate (P_i/PO_4^{3-}) linked by phosphoanhydride bonds and is ubiquitous in all domains of life (Rao, Gómez-García, and Kornberg 2009; Brown and Kornberg 2004). The widespread presence of polyP is related to the diverse cellular functions, including phosphate and energy storage, sequestration of cations, membrane channel formation, regulation of enzyme activities, control of gene activity, and stress response (Kulakovskaya, Vagabov, and Kulaev 2012). Several bacteria, algae, yeast, and fungi can rapidly and selectively hyperaccumulate P_i and store it as polyP, a phenomenon described for more than 70 years (Plouviez et al. 2021; Delgadillo-Mirquez et al. 2016; Christ and Blank 2019; Vila, Frases, and Gomes 2022). Besides its physiological roles, polyP is of significant interest in numerous industrial applications due to its physicochemical properties (Kulakovskaya, Vagabov, and Kulaev 2012). PolyPs are nonflammable, biodegradable, and nontoxic. Therefore, they are used in fertilizers, flame retardants, or as food additives (Kulakovskaya, Vagabov, and Kulaev 2012; Younes et al. 2019). Some properties are also directly defined by the polyP chain length. For example, the reactivation of actomyosin in meat products is most pronounced with $polyP_2$. Moreover, short-chain

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Take-Away

- The commercially available fluorescence sensor JC-D7 was used to establish a fast, highly sensitive polyP detection assay tailored for yeast samples.
- First-time evaluation of JC-D7's robustness to various abiotic factors, including polyP counteracting metal cations and polyP extraction agents.
- Pre-screening of yeast strains and rapid classification based on JC-D7 polyP detection.
- Researchers and industry professionals can benefit from this powerful, easy-to-use method for detecting polyphosphates in yeasts and other organisms.

polyPs are mildly acidic and can be used as pH-buffering agents. Long-chain polyPs are highly hydrophilic and bacteriostatic, both are beneficial properties in the food industry (Kulakovskaya, Vagabov, and Kulaev 2012; Shen and Swartz 2010). Additionally, their cation complexing ability increases with longer chain lengths (Wazer and Campanella 1950).

Despite the importance of polyP in biology and industry, many research questions regarding polyP, such as the primary signal (s) for polyP hyperaccumulation across the domains of life or the distribution of polyP in different compartments such as the mitochondrion, the nucleus, and the vacuole, remain unanswered (Rao, Gómez-García, and Kornberg 2009; Brown and Kornberg 2004; Borghi and Saiardi 2023). One of the key challenges of polyP research is the limited number of easy-to-use and inexpensive methods for direct detection and investigation of polyP (Christ, Willbold, and Blank 2020; Bru et al. 2017). Currently, ^{31}P NMR is the analytical gold standard to identify and quantitatively determine polyP concentrations and molecular weight. However, ^{31}P NMR requires samples with high polyP concentrations, expensive instruments, and a low throughput (Godinot et al. 2016; Christ, Willbold, and Blank 2019). Recently, two enzymatic assays for the determination of the total polyP content and average chain length were developed (Christ, Willbold, and Blank 2019; Christ and Blank 2018a). Although these assays allow reliable and high throughput characterization, they both rely on elaborated enzymatic cascades to detect the single monomer or dimer of polyP via colorimetric and fluorometric signals, respectively. Therefore, polyP is not detected in its original molecular structure and specific enzymes are required. Another option to determine the total polyP content in samples is via low molecular weight fluorescent probes. For polyP detection, DAPI is the most popular fluorescence probe. DAPI/polyP complexes emit yellow-green fluorescence (maximum emission intensity at 550 nm), while DAPI/DNA complexes exhibit their emission maximum in the blue-white part at 475 nm (DAPI excitation at 360 nm) (Aschar-Sobbi et al. 2008). Despite their high affinity toward polyP, DAPI is known to interact with lipid inclusions (Gomes et al. 2013; Streichan, Golecki, and Schön 1990), nucleotides (Martin and van Mooy 2013), RNA (Martin and van Mooy 2013), and inositol phosphates (Kolozsvari, Parisi, and Saiardi 2014). Moreover, polyPs with a chain length below 15 P-subunits cannot be detected via DAPI (Diaz and Ingall 2010). Therefore, DAPI staining–based detection is limited due to interferences with sample-related materials and by not covering the whole range of polyP chain lengths.

Angelova et al. (2014) introduced the fluorescent probe JC-D7 to specifically detect polyP in vitro as well as endogenous polyP in mammalian tissue sections. In these studies, JC-D7 exhibited high selectivity toward polyP and no responsivity to a wide range of ubiquitous cellular phosphate-containing molecules such as DNA, RNA, ATP, and GTP. Later on, Zhu et al. (2020) applied the dye for plant polyP detection (Zhu et al. 2020), and just recently, the sensor has found application as a polyP dye in environmental algae and bacteria (Yang et al. 2024). To our knowledge, JC-D7 has not yet been described for use in polyP detection in microbes used in biotechnology and food technology, including yeasts (Demling et al. 2024).

Here, we present the transfer of the protocol previously published by Angelova et al. (2014) to extracts of *Saccharomyces cerevisiae*. Baker's yeast is particularly intriguing for polyP research, given its capability to accumulate up to 28% (w/w) polyP (as KPO_3) per cell dry weight (Christ and Blank 2019). The impact of polyP concentration and chain length differences, polyP ligands such as metal cations, and polyP extraction agents on the fluorescence of JC-D7 was investigated. With the assay in hand, yeast strains carrying gene deletions in (poly)phosphate homeostasis were characterized.

2 | Material and Methods

2.1 | Chemicals, Materials, and Strains

The chemically produced sodium polyPs named Budit 4, 7, and 9 were obtained from Budenheim (Budenheim, Germany). The Budit number indicates the pH of a 1% (w/v) polyP solution. Budit 4 has a chain length of 20−25 P-subunits, Budit 7 of 10−15 P-subunits and Budit 9 of 4−6 P-subunits. Graham's salt (sodium polyphosphate, Emplura, lot 1.06529.1000) was obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and is reported in the publication of Robinson et al. (2022) with a chain length of $n = 22$ (Robinson et al. 2022). A 10 mg/mL stock solutions of Graham's and Budits were prepared by dissolving the powder in double-distilled water. For the polyP mix, equal volumes of the three polyP stock solutions (Budit 4, 7, and 9) were mixed to reach a final polyP concentration of 10 µg/mL. Ammonium polyPs (APPs) were obtained with chain lengths ranging from 7 up to 1472 P-subunits (Budenheim, Germany). Only APP8 dissolved readily in standard buffer (1 mM MOPS, 0.1 mM EDTA, pH 7) at 2 g/L. NaCl (240 mM) was added as dissolution aid to 2 g/L of the other APPs. JC-D7 dye was from Hölzel Diagnostic (Cologne, Germany). 5.9 mg/mL stock solutions of JC-D7 were prepared by dissolving the powder in pure DMSO. *S. cerevisiae* VH2.200 was a kind gift from the research institute of baker's yeast (Berlin, Germany), the laboratory strain CEN.PK 113-7D (Nijkamp et al. 2012) was obtained from Peter Kötter (Johann Wolfgang Goethe University Frankfurt, Germany). BY4742 MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (subsequently referred to as BY WT) was obtained from EUROSCARF (Oberursel, Germany). BY4742 strains with single deletions of YDR452W/PPN1 (subsequently referred to as BY ΔPPN1) and YFR034C/PHO4 (subsequently referred to as BY ΔPHO4) were selected from the

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EUROSCARF-collection (i.e., a single gene deletion *S. cerevisiae* library containing 4500 strains; http://www.euroscarf.de).

2.2 | PolyP-Rich Yeast Cells

PolyP–enriched yeast cells were generated according to (Christ and Blank 2019; for further details on polyP enrichment, see Christ and Blank 2019). The hyperaccumulation protocol started with a *S. cerevisiae* cultivation in a synthetically defined SD growth medium. In the second step, *S. cerevisiae* was starved in a synthetic medium containing all nutrients that are required for growth except for P_i (incubation time 6 h). Subsequently, P_i–starved *S. cerevisiae* was supplied with magnesium, glucose, and phosphate for 2.5 h to initiate polyP hyperaccumulation. The auxotrophic BY4742 strains were additionally supplied with the corresponding amino acids during preculture and starvation (Pronk 2002).

2.3 | Enzymatic PolyP Analytics

The polyP was extracted from polyP–rich *S. cerevisiae* cells with phenol/chloroform extraction according to (Christ and Blank 2018b). The Pi and polyP concentrations were measured colorimetrically with the Phosfinity kit from Aminoverse B.V. (Nuth, the Netherlands) (Christ and Blank 2018a).

2.4 | JC-D7 Fluorescence Assays

The JC-D7 polyP analytics in the absence of cells was performed according to (Angelova et al. 2014), unless stated otherwise. A reaction mixture of 200 μL was prepared by combining equal volumes of up to 50 μM JC-D7 diluted in assay buffer (20 mM HEPES buffer, pH 7.4, containing 1% (v/v) DMSO) and polyP solution (0–1000 μg/mL) in a 96-well polystyrene microtiter plate. Following 30 min incubations at 30°C, fluorescence measurements were performed at ambient temperatures in a Tecan Infinite M200 Reader (Tecan Austria GmbH, Grödig/Salzburg, Austria; excitation at 390 nm with a bandwidth of 9 nm, emission at 530 nm with a bandwidth of 20 nm, and detector gain 50).

The effects of detergents and inorganic metal salts on polyP–mediated JC-D7 fluorescence were evaluated by preincubating 50 μL of the polyP chain length mix (10 μg/mL polyP) with 50 μL of the fourfold concentrated test substances (individual substances prediluted in double-distilled water) for 30 min at 30°C before JC-D7 treatment (addition of 100 μL 50 μM JC-D7; 30 min incubation at 30°C) and fluorescence measurement. To investigate the effect of the metal chelator EDTA, the metal salt–pretreated polyP mixtures (final concentration of inorganic metal salt solutions 1 mM) were post-treated with 1 mM EDTA for 30 min, followed by JC-D7 addition and fluorescence readout.

S. cerevisiae extracts (preparation according to Christ and Blank 2018b) used in this work were diluted 1:500 in the assay buffer. Afterward, 100 μL of the respective dilution were mixed with 100 μL JC-D7 (20 μM) for 30 min at 30°C and fluorescence was measured in an Agilent BioTek Synergy Mx (Agilent Technologies, Santa Clara, USA; excitation at 390 nm with a bandwidth of 9 nm, emission at 530 nm with a bandwidth of 9 nm, automatic gain adjustment).

2.5 | Statistical Analyses

The statistical significance of experimental results was calculated by GraphPad Prism software version 8.02 (GraphPad Software Inc., CA, USA) using the tests indicated in the respective figure legends.

3 | Results

With the overall goal of developing a simple polyP detection method for assessing bio-polyP in *S. cerevisiae* samples, we investigated the suitability of the fluorescent dye JC-D7 as a sensor. For that purpose, industrial polyPs and polyPs from yeast origin were analyzed under various conditions with JC-D7.

3.1 | JC-D7 Response to Chemically Produced PolyPs

First, the dependence of JC-D7 fluorescence signal to polyPs with increasing concentrations and average chain lengths from 4 to 1500 P-subunits was examined in an experimental setting similar to that used by (Angelova et al. 2014).

The fluorescence signal of JC-D7/polyP increased with increasing concentration and chain length of the polyP standards, reaching values up to 40-fold higher than buffer-treated controls (Figure 1 and Supporting Information S1: Figures 1 and 2). JC-D7 did not respond to P_i (Figure 1 and Supporting Information S1: Figures 1 and 2), even in the presence of cell-relevant concentrations of up to 50 mM (data not shown). PolyPs with an average chain length between 4 and 1472 P-subunits could be detected, although at the lowest test concentrations of 0.5 and 1 μg/mL polyP, the signal was low (1.3–4.4-fold higher compared to control). For polyP concentrations higher than 10 μg/mL and average chain lengths above 79 P-subunits, JC-D7 fluorescence was saturated. A two-way ANOVA was used to estimate how the mean of JC-D7 fluorescence value changes according to the two independent parameters concentration and average chain length (Supporting Information S1: Table 1). A statistically significant difference in JC-D7 fluorescence was observed for both polyP concentration ($F = 151.9$, $p < 0.001$) and chain length ($F = 24.7$, $p < 0.001$), as well as for their interaction ($F = 2.2$, $p < 0.001$). The interaction effects and the saturation of JC-D7 fluorescence at certain polyP concentrations make reliable polyP quantification in heterogeneous chain composition samples impossible. Nevertheless, the fluorescent probe allows a semi-qualitative evaluation of polyP.

3.2 | Potential Abiotic Disturbance Factors Influencing JC-D7 Measurements

Due to its anionic nature, polyP can bind and sequester cations. This P_i backbone masking might affect JC-D7's interaction with polyP. Thus, different metal salt solutions were analyzed with

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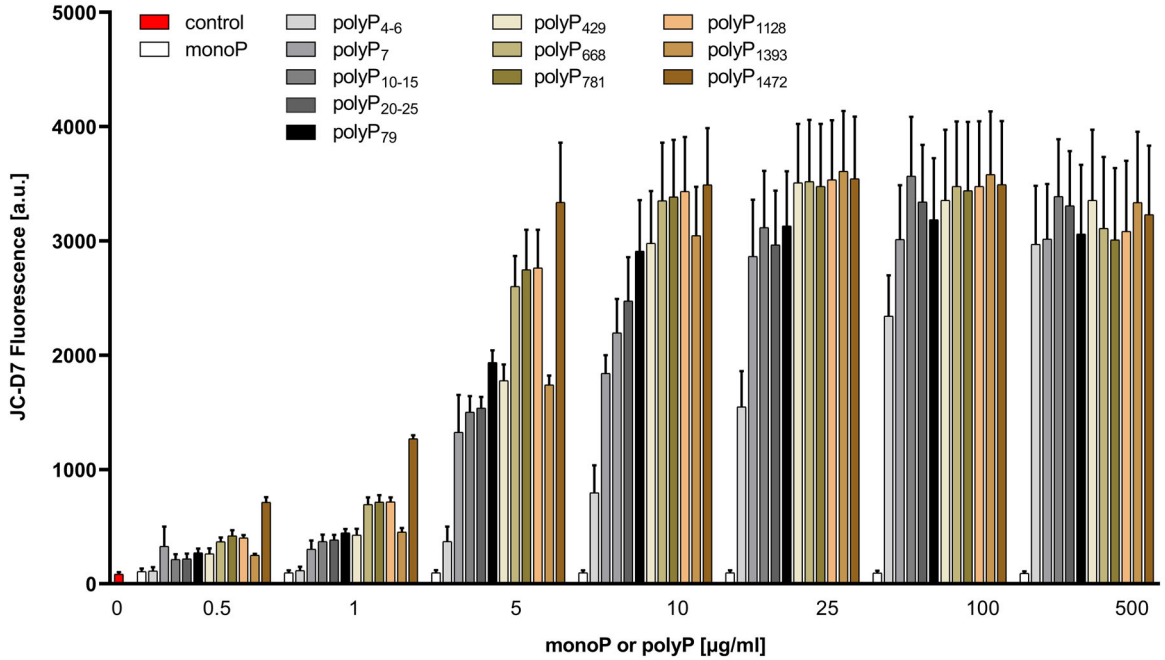

FIGURE 1 | Polyphosphate concentration and chain length dependence of the JC-D7 fluorescence signal. A measure of 25 µM solutions of JC-D7 was combined with increasing quantities of monoP or polyP of different chain lengths, followed by fluorescence detection ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 530$ nm; Tecan Infinite M200 Reader). Data are presented as means ± SEM of three independent experiments.

respect to their effects on JC-D7 polyP response (Figure 2 and Supporting Information S1: Figure 3).

Treatment with EDTA before the addition of JC-D7 reduced the quenching of the fluorescence for some of the metal solutions (Figure 3). These results should be considered if JC-D7 is used to assess polyP in samples containing macro- and micro-minerals, such as yeasts or cells/tissues from other unicellular or multicellular organisms.

In the case of JC-D7 usage on cell extracts, molecules provided by the cell extraction buffers may also have an impact on the interaction of polyP and JC-D7. Hence, the effect of various agents, commonly used for polyP extraction from yeast biomass (Christ and Blank 2018b), on the fluorescence outcome was tested (Figure 4). A polyP mix was pretreated for 30 min with different concentrations of either NaCl, LiCl, Triton X-100, sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), and Pluronic F-127, followed by fluorescence detection with 25 µM JC-D7. There was a decrease in fluorescence signal after ≥ 0.1% SDS treatment. All other solvents and salt solutions had no significant effect on the fluorescence.

3.3 | Validation of Polyp Detection With JC-D7 in PolyP–Rich *S. cerevisiae* Extract

Even under various experimental conditions, JC-D7 could not be loaded into intact yeast cells for direct measurement of intracellular polyP (data not shown). Instead, the JC-D7 reaction with extracts from polyP–enriched *S. cerevisiae* VH2.200 cells ($VH2.200_{fed}$) was compared with those of $polyP_{20–25}$ (Budit 4), $polyP_{10–15}$ (Budit 7), $polyP_{4–6}$ (Budit 9) (either at 2.5 or 5.0 µg/mL) standards (Supporting Information S1: Figure 4). JC-D7 fluorescence analysis at 530 nm, as depicted by Angelova et al. (2014), is also applicable for polyP detection in yeast extracts. PolyP Budit spectra match yeast extract spectra, suggesting that no other sample-related components interfere with JC-D7 fluorescence detection. The spectra also demonstrate an increase in signal intensity with the chain length as well as with the concentration of the respective Budit standard (2.5 vs. 5.0 µg/mL), yet not in a proportional manner as observed before (Figure 1). It is possible to analyze yeast samples with low fluorescent signal intensity using the fluorescent dye because the intensity of the lowest Budit spectra ($polyP_{4–6}$) is 2.8-fold higher compared to the control (buffer). Thus, JC-D7 is suitable for semi-quantitative assessment of chemically produced polyP as well as bio-polyP in *S. cerevisiae* extract even at low concentrations.

A series of measurement parameters, including temperature, pH value, and incubation time, were tested to optimize the JC-D7 fluorescence staining protocol (Figure 5). These parameters have previously been demonstrated to be critical for fluorescence measurements (Hope et al. 2016). Therefore, extracts of the industrial yeast VH2.200 and the laboratory strain CEN.PK 113-7D either after P_i starvation or P_i feeding were applied. Considering the temperature during the JC-D7 treatment period, no differences in the relative fluorescence intensity were observed between 24°C and 30°C (Figure 5A). However, a labeling temperature of 37°C led to a minor decrease in fluorescence. Consequently, 30°C was used for further JC-D7 incubations. Besides that, the effect of temperature variations inside the plate reader during fluorescence measurement was investigated (24°C, 30°C, and 37°C) (Figure 5B). In general, the relative fluorescence intensity readout was highest at 30°C. However, here only differences with *S. cerevisiae* VH2.200 extract, and $polyP_{10–15}$ (Budit 7) were detected (Figure 5B). Next, the influence of the pH value on the fluorescence intensity of JC-D7 was investigated (Figure 5C). Yeast extracts and chemical polyP standards were diluted in buffers with pH values ranging from 3.0 to 9.0. A

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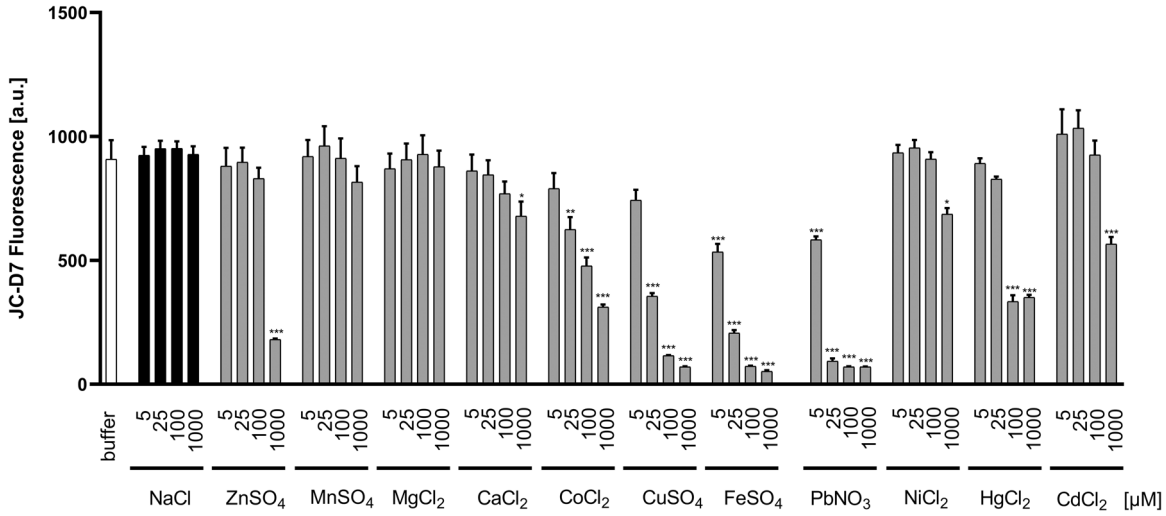

FIGURE 2 | PolyP–mediated JC-D7 fluorescence in the presence of inorganic metal salts. A measure of 10 µg/mL of polyP mix was pretreated with either assay buffer or increasing quantities of metal salt solutions for 30 min, followed by detection of JC-D7 fluorescence emission (25 µM JC-D7; $\lambda_{ex} = 90$ nm, $\lambda_{em} = 530$ nm; Tecan Infinite M200 Reader). Data are presented as means ± SEM of three independent experiments. Significant differences from buffer incubations are marked by asterisks (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; one-way ANOVA with Dunnett's post hoc test).

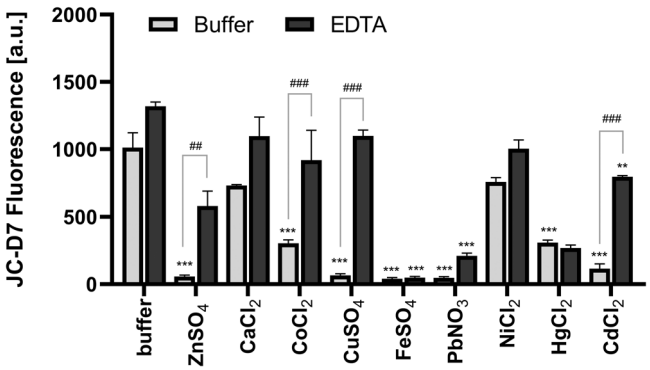

FIGURE 3 | The effect of EDTA on metal salt quenching of polyP JC-D7 fluorescence. A measure of 10 µg/mL of polyP mix was pretreated with 1 mM metal salt solutions, followed by buffer treatment or 1 mM EDTA posttreatment and fluorescence detection with 25 µM JC-D7 ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 530$ nm; Tecan Infinite M200 Reader). Data are presented as means ± SEM of three independent experiments. Significant differences from buffer incubations (**$p < 0.01$ and ***$p < 0.001$; two-way ANOVA/Tukey's post hoc test) or from metal salt treatment ($^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$; two-way ANOVA/Tukey's post hoc test) are indicated.

fluorescence signal was measurable irrespective of the pH value of the test solution. However, the JC-D7 signal decreased for samples containing cell extract at the pH values of 3.0 and 9.0. Therefore, solutions with these two pH values should be avoided to prevent a substantial drop in the fluorescence signal. In our setup, the pH value was kept around 7.4 using HEPES buffer. Moreover, the incubation time after JC-D7 addition was examined (Figure 5D). Samples were mixed and the JC-D7 signals were determined immediately ($t = 0$ min) and after different time periods. Incubation times of 10 min resulted in fluorescence intensities almost comparable to those measured directly. However, after 20 min incubation, the fluorescence values of cell extract samples and chemically produced polyP standards slightly decreased and continued to decline after 30 min incubation. The cause of the signal decline after 20 min remains ambiguous and necessitates additional exploration. Based on these findings, it is advisable to conduct measurements on JC-D7-treated samples promptly or within a 20-min timeframe to mitigate potential signal reduction. As the JC-D7 evaluation methodology shows stability under different test conditions, it can be used for the evaluation of a broad spectrum of chemical polyP samples, polyP–containing yeast cell extracts, and probably also extracts prepared from other prokaryotic and eukaryotic cells or tissues.

Finally, the suitability of JC-D7 to monitor yeast samples of different polyP concentrations was assessed. Therefore, extracts of yeast strains with varying polyP accumulation capabilities were treated with JC-D7 (Figure 6A) and the results compared to the established, enzymatic assay for total polyP quantification applying *S. cerevisiae* exopolyphosphatase scPpx1p and inorganic pyrophosphatase scIpp1p (Figure 6B) (Christ and Blank 2018a).

In the enzymatic assay, VH2.200$_{fed}$ exhibited a polyP content of 26.8% (w/w, as KPO_3), whereas CEN.PK 113-7D$_{fed}$ produced 4.3% (w/w) polyP and BY WT$_{fed}$ produced 7.5% (w/w) polyP. However, by deleting the endo- and exopolyphosphatase Ppn1p (Sethuraman, Rao, and Kornberg 2001), BY ΔPPN1 increased the polyP production up to 10.3% (w/w) (Figure 6B) (Pestov, Kulakovskaya, and Kulaev 2005; Andreeva et al. 2016). In BY ΔPHO4, deleted in the transcriptional activator Pho4p (Lemire et al. 1985), the polyP content was markedly lower (2.9%, w/w). Under phosphate limitation, Pho4p activates transcription of Pho-responsive genes together with Pho2p, leading to the expression of Pho regulon genes, including phosphate transporters, phosphatases, and vacuolar storage regulators. These genes play a critical role in the P_i import process and in maintaining intracellular P_i levels (Bun-Ya et al. 1991; Ogawa, DeRisi, and Brown 1995, 2000). Applying JC-D7 to these *S. cerevisiae* extracts, the fluorescence data (Figure 6A) matched the trend of the enzymatic polyP data. The strain-related differences in polyP levels were clearly depicted via fluorescence measurement (Figure 6A). Therefore, one can assess and categorize

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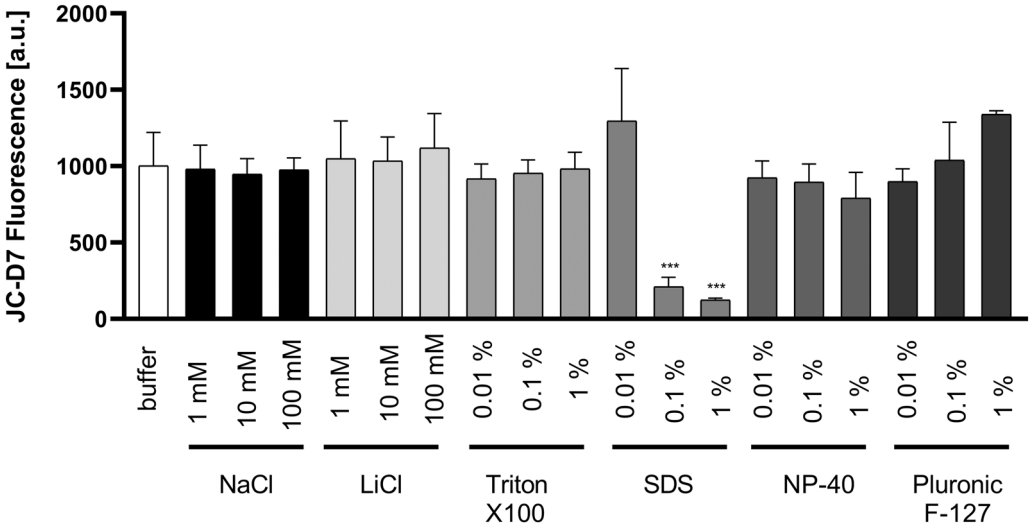

FIGURE 4 | Effect of common polyP extraction agents on polyP JC-D7 fluorescence. A measure of 10 μg/mL of a polyP mix was pretreated with either assay buffer or increasing quantities of yeast polyP extraction agents for 30 min, followed by fluorescence detection with 25 μM JC-D7 ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 530$ nm; Tecan Infinite M200 Reader). Data are presented as means ± SEM of three independent experiments. Significant differences from buffer incubations are marked by asterisks (***$p < 0.001$; one-way ANOVA with Dunnett's post hoc test).

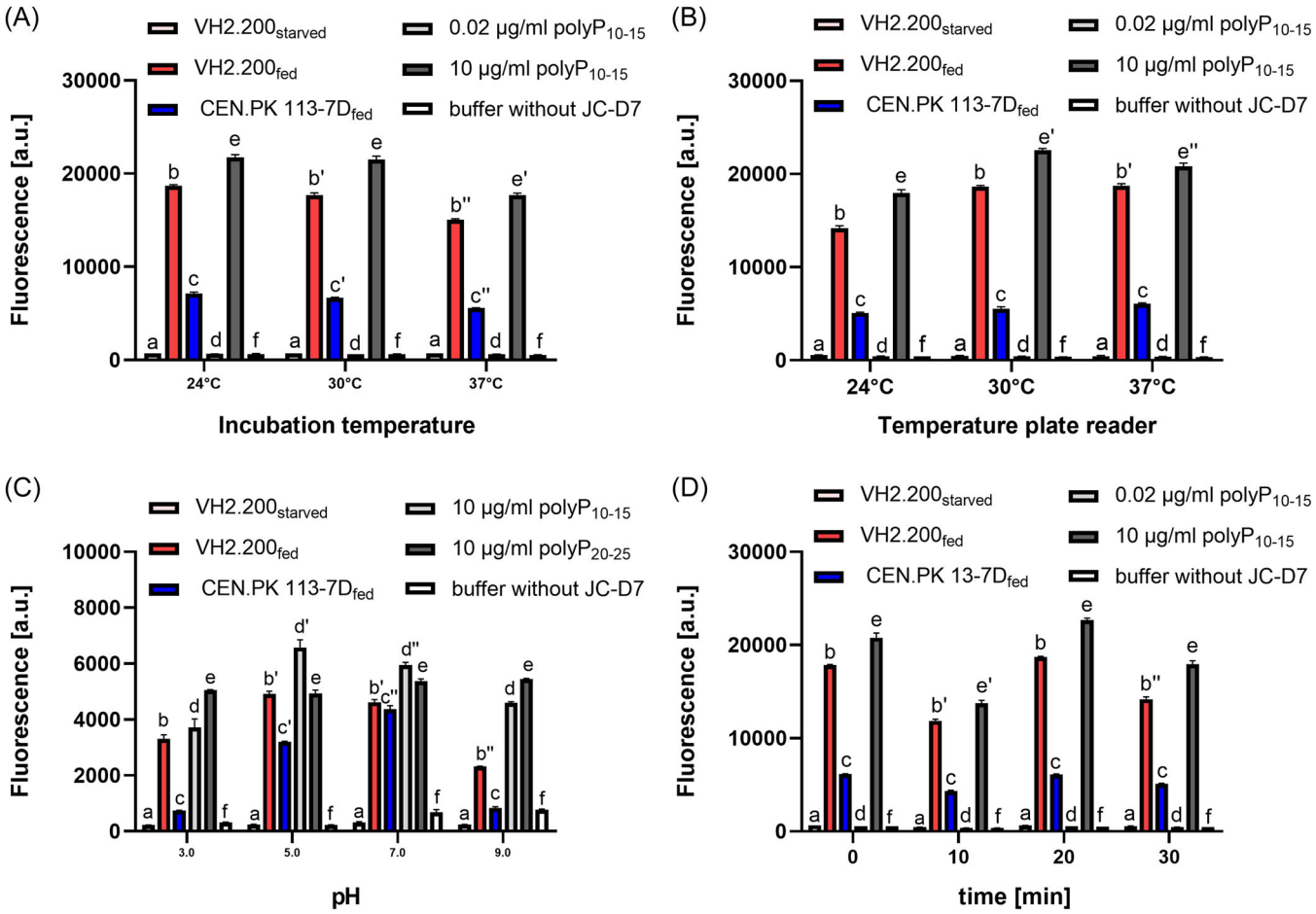

FIGURE 5 | Optimization of JC-D7 application on yeast extracts. PolyP standards as well as extracts from P_i-starved or P_i-fed yeasts were treated with 10 μM JC-D7, followed by fluorescence detection ($\lambda_{ex} = 390$ nm, $\lambda_{em} = 530$ nm; Agilent BioTek Synergy Mx). The impact of the (A) incubation temperature during JC-D7 staining; (B) temperature of the plate reader during readout; (C) pH of the JC-D7 staining buffer; and (D) duration of incubation (all time points mixing via pipetting) after adding JC-D7 dye was analyzed. Data are presented as means ± SEM of three independent experiments. (A–D) The effect of the different parameters in the respective panels was analyzed with one-way ANOVA/Tukey's multiple comparison test ($p < 0.05$). Significant differences within one panel are indicated by not sharing the same letters. $VH2.200_{starved}$ (a); $VH2.200_{fed}$ (b, b′, b″); $CEN.PK\ 113\text{-}7D_{fed}$ (c, c″); 0.02 μg/mL $polyP_{10–15}$ (d, d′, d″); 10 μg/mL $polyP_{10–15}$ (e, e′, e″); 10 μg/mL $polyP_{20–25}$ (e); buffer without JC-D7 (f).

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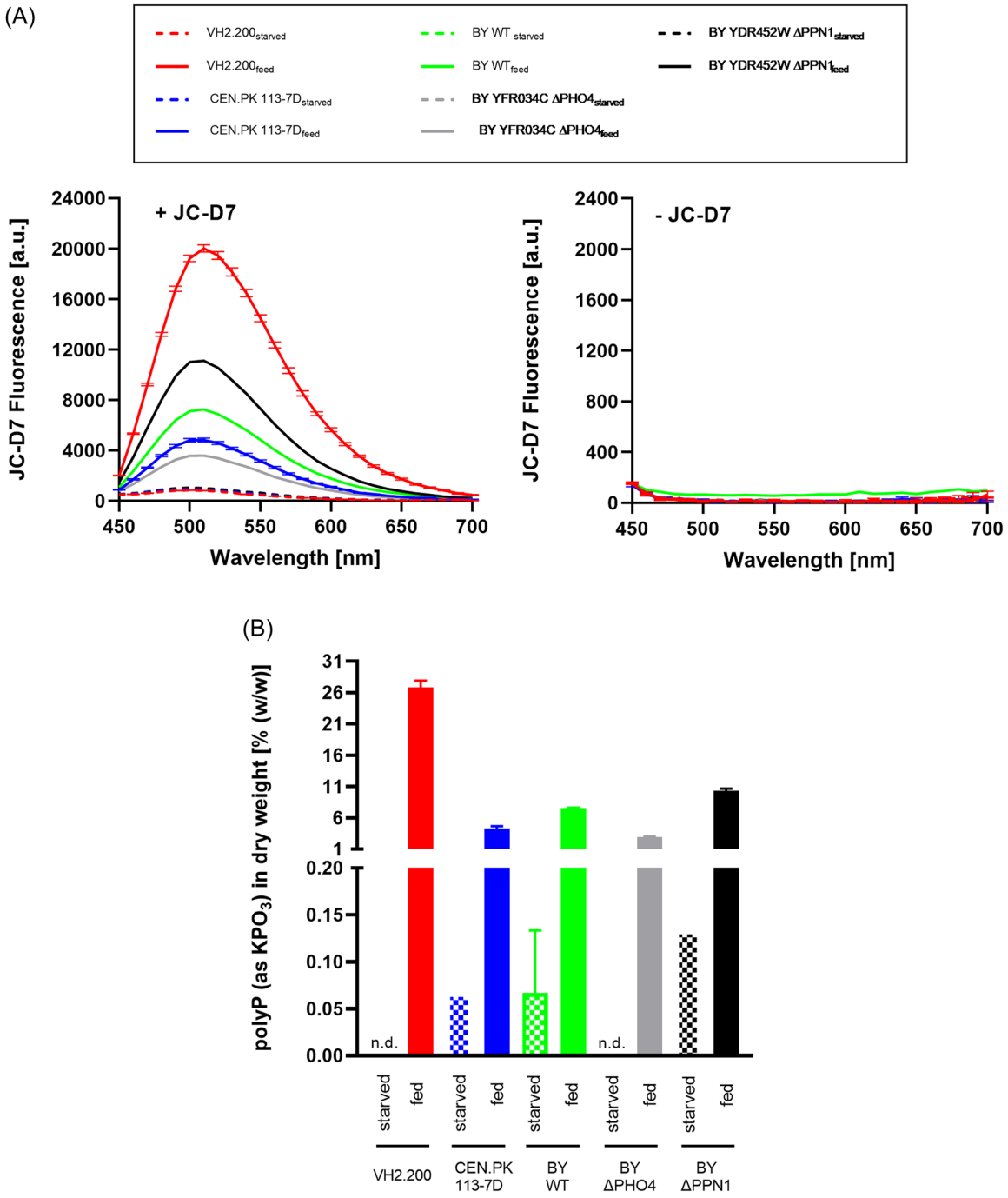

FIGURE 6 | Comparison of JC-D7 fluorescence measurements and enzymatic analysis of polyP in yeast extracts. (A) Extracts from phosphate-starved or phosphate-fed yeasts were treated either with 10 μM JC-D7 or buffer (temperature 30°C, time 30 min), followed by detection of fluorescence emission spectra (λ_{ex} = 450–700 nm). (B) The same extracts were analyzed for their polyP content by enzymatic assays. Data are presented as means ± SEM of three independent experiments.

polyP production of various yeast strains based on the JC-D7 fluorescent signal.

4 | Discussion

In our work, the protocol for polyP detection published by Angelova et al. (2014) was applied to *S. cerevisiae* extract samples. After validating the capability of JC-D7 for detecting chemically synthesized polyPs with varying chain lengths, we examined the influence of potential interfering abiotic factors and parameters originating from yeast pretreatment or yeast extract preparation procedure (metal cations, extraction agents, pH, and temperature). Finally, JC-D7 was tested as a polyP fluorescence dye on various yeast cell extracts including two *S. cerevisiae* strains of the EUROSCARF collection containing single gene deletions in polyP metabolism.

The detection of polyP at various concentrations and chain lengths was demonstrated, and P_i up to 50 mM did not interfere

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with JC-D7 measurements. This is in line with the results of Angelova et al. (2014). Usually, the P_i concentration in yeast is between 10 and 75 mM (van Eunen et al. 2010; Bru et al. 2016) so the P_i content in yeast samples should not impact the JC-D7-based detection of polyP. A 5.0- to 15.0-fold increase in JC-D7 fluorescence upon treatment with 10 μg/mL polyP of 15, 60, or 120 P-subunits chain length was demonstrated by Angelova et al. (2014). As an additional measure, $polyP_{60}$ was saturating the sensor at a concentration of 50 μg/mL in the same study (Angelova et al. 2014). We observed a stronger fluorescence response of JC-D7 in the mentioned concentration and chain length range. Moreover, fluorescence saturation was achieved already at 10 μg/mL for polyP molecules ≥ 79 P-subunits. Similar to Angelova et al. (2014), longer polyP chains had a stronger fluorescence response. However, in contrast to the minimum detection limit of 2 μg/mL polyP given by Angelova et al. (2014), the outcome of our plate reader fluorescence measurements revealed a response even in the presence of 0.5 μg/mL polyP. Our statistical evaluation showed that polyP amount and chain length as independent variables are decisive for the JC-D7 fluorescence outcome, but also the combined effects of these two factors. This finding is important since biological samples usually contain a mixture of diverse polyP molecules with a high polydispersity (Christ et al. 2020; Breus et al. 2012; Khourchi, Delaplace, and Bargaz 2023; Andreeva et al. 2016), which makes quantification challenging (Christ, Willbold, and Blank 2019; Christ and Blank 2018a). However, as a semi-quantitative parameter, JC-D7 fluorescence is suitable for evaluating those samples. Christ and Blank (2019) have recently introduced two consecutive enzymatic assays designed for the quantitative determination of both total polyP concentration and the average chain length within biological samples. These assays determine the total polyP content via phosphate standards in the range from 20 to 200 μM P_i and the concentration of the polyP chains (for the average chain length calculation) in the range of 1–10 μM via $polyP_2$ standards (Christ, Willbold, and Blank 2019). Within our study's data set, the JC-D7 fluorescence and the results of the enzymatic assay showed a consistent trend for each *S. cerevisiae* extract analyzed. Thus, both assays allow to record polyP molecules, but the JC-D7 test cannot quantify them. The lowest applied concentration of Budit 7 in the JC-D7 assay (0.5 μg/mL) translates to 4.0 μM P_i in the total polyP assay (Christ, Willbold, and Blank 2019) (see Supporting Information S1: 1). Thus, measurements with JC-D7 are slightly more sensitive than enzymatic polyP detection. When deciding which of the two detection methods should be used for polyP assessment, the sample quantity and the research question must be considered.

Another fluorescence dye often used for polyP detection in cells and tissues is DAPI (Christ, Willbold, and Blank 2020). DAPI is most prominently known for its application in DNA detection (450–475 nm emission maximum upon binding to dsDNA) (Aschar-Sobbi et al. 2008; Kapuscinski 1990, 1995) but it is also applicable to the detection of polyP by switching the emission wavelength to 525 nm (Aschar-Sobbi et al. 2008). Thus, DAPI is not specific for polyP, as is the case for JC-D7 (Angelova et al. 2014). DAPI detects polyP only with a chain length greater than 15 P-subunits; short-chain polyPs (e.g., 3–5 P-subunits) react slightly or not at all with DAPI at polyP concentrations ranging from 0.5 to 3 μM P_i (Aschar-Sobbi et al. 2008; Diaz and Ingall 2010). Likewise, the PPK, PPX, and TBO quantification methods described by Ohtomo et al. (2008), as well as the affinity polyP labeling approach using a recombinant polyP binding domain reported by Saito et al. (2005) (Saito et al. 2005) were not suitable for detecting short-chain polyPs. In contrast to JC-D7, no difference in the DAPI fluorescence intensity was registered following treatment with 15–130 P-subunit polyP standards between 1.5 and 3 μM P_i (Diaz and Ingall 2010). However, Aschar-Sobbi et al. (2008) demonstrated a slower kinetic in DAPI-polyP fluorescence increase for polyP with an average chain length of 15 P-subunits compared to 130 P-subunits. The rate was suggested as a polyP chain length indicator, but this principle was only demonstrated for the two named chain lengths varying by 115 P-subunits and a concentration of 0.5 μg/mL Aschar-Sobbi et al. (2008). Similar to JC-D7, polyP–induced DAPI fluorescence very likely has independent contributors, making accurate quantification challenging. A nearly linear dependency of the DAPI signal on the polyP concentration has been demonstrated between 25 and 200 ng $polyP_{75}$/mL. This linear relationship is maintained until 1.5 μg/mL polyP. A signal saturation is registered at higher concentrations Aschar-Sobbi et al. (2008). This offers a higher sensitivity, but also lower saturation levels compared to the JC-D7 fluorescence assay.

The sensitivity of JC-D7 is suitable for the envisioned application with yeast extracts since the enzymatically determined concentration via total polyP assay (Christ and Blank 2018a) of the low polyP-producing strain is at ~2 mM P_i and of the high-producing strain at ~45 mM P_i Aschar-Sobbi et al. (2008) and Diaz and Ingall (2010) both reported a decrease in polyP–induced DAPI fluorescence in the presence of ≥ 10 mM calcium or a salinity level of 150 mM for chains of around 15 P-subunits, respectively (Aschar-Sobbi et al. 2008; Diaz and Ingall 2010). JC-D7 response was not affected by 100 mM NaCl, LiCl, $MgSO_4$, or $CaCl_2$ within our experimental setup. According to Angelova et al.'s (2014) study, the polyP responsiveness of JC-D7 was substantially reduced in 150 mM KCl saline buffers. Furthermore, a very recent manuscript by Yang et al. (2024) revealed that concentrations exceeding 100 mM NaCl had a negative impact on JC-D7 fluorescence. Therefore, keeping the ionic strength in the detection solution as low as feasible is recommended to make the JC-D7–based fluorescence assay highly sensitive. A diminishing effect of NaCl concentrations as low as 5 mM on polyP outcome was also observed in the extraction protocol published by Christ and Blank (2018b). It might be caused by reduced extraction efficiencies and/or by an inhibitory effect on polyP detection. A determination of which factor caused the inhibition was not attempted in the aforementioned study. However, this should be considered during sample preparation, as extraction and detection are inevitably linked.

Amongst the detergents tested, only SDS > 0.1% (v/v) had a negative effect on the polyP–induced JC-D7 fluorescence. A negative effect of SDS was also noted during the optimization of analytical polyP extraction by Christ and Blank (2018b). When adding 2.8% (v/v) SDS into the lysis buffers for polyP extraction, polyP quantification was completely inhibited. SDS breaks ionic bond interactions and hydrophobic interactions, as well as hydrogen bonds, when interacting with proteins (Hou, He, and Wang 2020). This might among other things have led to

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denaturation and thus inactivation of the enzymes applied for polyP quantification by Christ and Blank (2018). The detergent may have impaired the interaction between JC-D7 and polyPs as well. Future studies will require an in-depth analysis to determine the so far unknown mechanism by which JC-D7 interacts with polyP, to provide more insight into its favorability for polyP detection.

The JC-D7 measurement and enzymatic polyP quantitation in yeast extracts showed similar patterns in this study. The above-average polyP accumulation capacity of *S. cerevisiae* VH2.200 (Christ and Blank 2019; Fees et al. 2023) was demonstrated by both methods. Moreover, yeast mutants previously identified to hypoaccumulate (gene deletion mutant of transcriptional activator Pho4p/YFR034C) or hyperaccumulate (gene deletion mutant of polyphosphatase Ppn1p/YDR452W) polyP (Andreeva et al. 2016; Ogawa, DeRisi, and Brown 2000; Lonetti et al. 2011) by means of enzymatic quantification could be verified with the JC-D7 fluorescence assay. In the future, the JC-D7 fluorescence assay might be used for high-throughput screening of various engineered and natural yeast strains. As a result of these findings, yeasts with more efficient biotechnological polyP production reusing P_i-containing wastewater or side streams can be identified (Fees et al. 2023; Blank 2023).

PolyP is also a polyelectrolyte that allows metal cations to complex with negatively charged P_i moieties. Storage of polyP inside microorganisms enables the bioaccumulation of other minerals and trace elements. In fact, metal–polyP complexes play a key role in the sequestration and detoxification of metals, which so far has mostly been studied in prokaryotic and eukaryotic microorganisms (Sanz-Luque, Bhaya, and Grossman 2020; Kulakovskaya 2018; Sun et al. 2022). Monovalent (Na^+ and Ag^+), divalent (Ca^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}), and trivalent cations (Fe^{3+}, Al^{3+}, and Eu^{3+}) are reported to interact with polyP (Müller, Schröder, and Wang 2019; Jastrzab et al. 2021; Park et al. 2021). PolyP can bind metal ions with dissociation constants in the micromolar to nanomolar range (Wazer and Campanella 1950), which agrees well with the concentrations and phosphate/element ratio reported for yeasts (Cockrell et al. 2011; Nguyen, Dziuba, and Lindahl 2019). Moreover, complex formation is largely determined by the coordination sphere, with longer chains and more coordination numbers conducive to the complex formation (Wazer and Campanella 1950; Müller, Schröder, and Wang 2019; Park et al. 2021). According to our results, polyP–induced JC-D7 fluorescence can change significantly after treatment with metal salt solutions. $FeSO_4$, $CuSO_4$, $CoCl_2$, $ZnSO_4$, $PbNO_3$, and $HgCl_2$ showed the most pronounced quenching effects. The conformation of polyP could be altered by metal ions (Müller, Schröder, and Wang 2019; Park et al. 2021; Schröder et al. 2022), which also might impact its subsequent interaction with the JC-D7 fluorescence sensor. Based on Yan et al.'s (2023) findings, Zn^{2+} reduces the stability of the P–O–P bond by causing a conformational change in polyP due to chelation, which in turn promotes hydrolysis. Under the conditions chosen in the present study, the latter point does not seem to matter much, because the addition of the metal chelator EDTA, at least for zinc, could restore JC-D7's fluorescence. To counteract metal quenching in JC-D7–based polyP assessments of zinc–enriched cell/tissue samples, membrane-permeable chelators such as Tris(2-pyridylmethyl)amine (TPA) or *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) might be used to trap biologically mobile zinc (Huang et al. 2013). Besides zinc, both chelators also bind other divalent elements such as cadmium, cobalt, nickel, and copper (Anderegg et al. 1977), and are thus promising candidates for eliminating such polyP–metal complexes.

5 | Conclusion

This study presents the so far unprecedented application of JC-D7 for the detection of biologically produced polyPs extracted from *S. cerevisiae*. The robustness of the JC-D7 signal against a variety of abiotic factors and extraction agents was demonstrated. The semi-quantitative nature and the high sensitivity of the JC-D7 assay would allow its application in an envisioned workflow for pre-screening a large number of yeast strains and rapid classification of these strains concerning their polyP production capabilities. Thus, yeast polyP accumulation could be studied further, and the results used to make bio-polyP.

Author Contributions

Alexander Deitert, Claudia Keil, and Jana Fees conceived the study. Duc Nguyen Van and Anna Mertens performed most experiments. Duc Nguyen Van and Claudia Keil carried out the analysis of the abiotic disturbance factors and the investigation of JC-D7 response to chemically produced polyPs. Alexander Deitert, Anna Mertens, and Jana Fees carried out the analysis of polyP staining with yeast extracts. Alexander Deitert, Claudia Keil, Jana Fees, Maria Maares, Hajo Haase, and Lars Mathias Blank wrote the manuscript.

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Conflicts of Interest

Lars Mathias Blank filed two patent applications: "Zusammensetzung, enthaltend getrocknetes Polyphosphat und Verfahren zur Gewinnung von Polyphosphat aus polyphosphat-haltigen Hefezellen dazu" (DE 10 2019 131 561.1) and "Polyphosphatreiche Hefeextrakte und Herstellverfahren dazu" (DE 10 2018 130 081.6, PCT/EP2019/082709). The other authors declare no conflicts of interest.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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