Biotechnological synthesis of water-soluble food-grade polyphosphate with *Saccharomyces cerevisiae*

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**Funding information**
National Heart Lung and Blood Institute of the NIH, Grant/Award Number: R35 HL135823; Deutsche Bundesstiftung Umwelt, Grant/Award Number: 33006/01

**Abstract**
Inorganic polyphosphate (polyP) is the polymer of phosphate. Water-soluble polyPs with average chain lengths of 2–40 P-subunits are widely used as food additives and are currently synthesized chemically. An environmentally friendly highly scalable process to biosynthesize water-soluble food-grade polyP in powder form (termed bio-polyP) is presented in this study. After incubation in a phosphate-free medium, generally regarded as safe wild-type baker’s yeast (*Saccharomyces cerevisiae*) took up phosphate and intracellularly polymerized it into 26.5% polyP (as KPO₃, in cell dry weight). The cells were lyzed by freeze-thawing and gentle heat treatment (10 min, 70°C). Protein and nucleic acid were removed from the soluble cell components by precipitation with 50 mM HCl. Two chain length fractions (42 and 11P-subunits average polyP chain length, purity on a par with chemically produced polyP) were obtained by fractional polyP precipitation (Fraction 1 was precipitated with 100 mM NaCl and 0.15 vol ethanol, and Fraction 2 with 1 final vol ethanol), drying, and milling. The physicochemical properties of bio-polyP were analyzed with an enzyme assay, ³¹P nuclear magnetic resonance spectroscopy, and polyacrylamide gel electrophoresis, among others. An envisaged application of the process is phosphate recycling from waste streams into high-value bio-polyP.

**KEYWORDS**
biosynthesis, food additive, natural products, polymers, polyphosphate

**INTRODUCTION**
Inorganic polyphosphate (polyPₙ) is the polymer of phosphate (P) and can be found in most living organisms. n is the number average degree of polymerization, which is referred to as “chain length” here, and can range from two to a thousand P-subunits (Harold, 1966; Kulaev, Vagabov, & Kulakovskaya, 2005; Rao, Gomez-Garcia, & Kornberg, 2009). Some literature does not regard polyP₂ as polyP due to the different physiological roles of polyP₂ and polyPₙ≥₃ in cell biology. From an industrial standpoint, all condensed polyPs (polyPₙ≥₂) are of interest and were, therefore, included in the term “polyP” and measured as part of the total polyP concentration in this study. The polyP structure is classified in linear, cyclic, and branched polyP, whereas micro-organisms produce only linear polyP. See Christ, Willbold, and Blank (2020) for a detailed review of the molecular polyP structure. Whereas alkaline earth metal salts of linear polyP are water-insoluble, alkali metal salts of linear polyP are water-soluble (Kulaev et al., 2005, p. 10; Van Wazer, 1958, p. 671). PolyP (referring to linear polyP from here on) is used as a food additive...
due to its beneficial physicochemical properties, whereas the chain length determines which properties are more pronounced. For example, polyP2 and 3 reactivate the water holding capacity of meat after the rigor mortis and intermediate-chain polyP complexes higher valent cations in soft cheese production.

The biotechnological synthesis of polyP appears promising because the polyP chain length in microorganisms can reach up to a thousand P-sub-units (Rao et al., 2009). Furthermore, microorganisms can produce polyP from impure P, whereas chemical polyP synthesis relies on pure P. We recently reported a process to obtain Saccharomyces cerevisiae (baker’s yeast) containing up to 28% polyP (as KPO3) in cell dry weight (Christ & Blank, 2019). S. cerevisiae was chosen as the polyP production host because it is generally regarded as safe (GRAS) and other food-related fungi, such as Pichia pastoris, Kluyveromyces lactis, and Hansenula saturnus, produced only little polyP (<7%) in our hands (data not shown).

In S. cerevisiae, the transport of P across the cell membrane is mediated by the low-affinity transporters Pho87 and Pho90 (Km ~1 mM) and two high-affinity P, transporters Pho84 and Pho89 (Km ~10 μM, Figure 1; Canadel, Gonzalez, Casado, & Arino, 2015). During polyP synthesis, S. cerevisiae primarily metabolizes glucose via alcoholic fermentation to ethanol, which produces both the required energy and activated P in the form of adenosine triphosphate (ATP; Christ & Blank, 2019). PolyP is synthesized by the vacuolar transporter chaperone (VTC). The enzyme complex is located in the vacuolar membrane, couples the synthesis of polyP to its translocation across the vacuolar membrane, consumes ATP, consists of the five subunits VTC 1–5, and is presumably dependent on the proton gradient that is created by the V-ATPase (Desfougeres, Gerasimaite, Jessen, & Mayer, 2016). Langen and Liss (1958) showed that, after P starvation, long-chain polyP is produced de novo, and later hydrolyzed to shorter chain polyP. The high polyP content during P feeding is due to an increased polyP synthesis and not due to reduced polyP degradation (Liss & Langen, 1962). The polyP degradation in the vacuole is facilitated by S. cerevisiae endopolyphosphatase 1 and 2 (Gerasimaite & Mayer, 2016, 2017). P that is generated in the vacuole is transported to the cytosol by Pho91 (Eskes, Deprez, Wilms, & Winderickx, 2018).

To the authors’ best knowledge, there are no reports on the biosynthesis of a food-grade water-soluble polyP with a highly scalable biotechnological process. The overall goal of the here presented study was the biosynthesis of polyP from P, with the focus on the purification (so-called preparative polyP extraction) of the synthesized polyP from polyP-rich S. cerevisiae. The desired characteristics of such a biotechnologically synthesized polyP included: appearance as a dry white water-soluble powder, food-grade quality, a linear molecular structure, a purity comparable with chemically produced polyP, and one polyP with mostly sodium and one with mainly potassium as the counterion. The term “bio-polyP” is proposed for the product of the here described synthesis. All process steps were designed to be highly scalable for intended large scale production. The physicochemical properties of the bio-polyP were analyzed and compared with chemically synthesized polyP.

### FIGURE 1 PolyP metabolism and regulation in Saccharomyces cerevisiae. The information content of the figure originates from these references Canadel et al. (2015); Gerasimaite et al. (2017); Goldt et al. (2017); Ogawa, DeRisi, and Brown (2000); Tomar and Sinha (2014); and Wild et al. (2016). Adh, alcohol dehydrogenase; ATP, adenosine triphosphate; Hxt, hexose transporter; Pdc1 and 5, pyruvate decarboxylase complex 1 and 5; Ppn 1 and 2, endopolyphosphatase 1 and 2; VTC, vacuolar transporter chaperone. [Color figure can be viewed at wileyonlinelibrary.com]

### 2 | MATERIALS AND METHODS

#### 2.1 | Chemicals, materials, and strains

PolyP Budit 4, polyP p100, and S. cerevisiae VH2.200 were kind gifts from the Chemische Fabrik Budenheim (Budenheim, Germany), Dr. Toshikazu Shiba (Regenetiss Incorporation, Higashi, Kunitachi, Tokyo, Japan), and the Research Institute for baker’s yeast (Berlin, Germany), respectively. PolyP from Roth was purchased from Carl Roth (Karlsruhe, Germany).

#### 2.2 | Synthesis of bio-polyP

NaCl and NaOH were used in Steps 10–12 to produce sodium bio-polyP. KCl and KOH were used to synthesize potassium bio-polyP. Nondenatured ethanol was used. All steps were performed at room temperature except Steps 1 and 6. 1. PolyP-rich S. cerevisiae was produced according to Christ and Blank (2019). Briefly, 0.625 g cell wet weight S. cerevisiae (strain VH2.200) was...
incubated anaerobically for 6 hr at 30°C in 1 L P_{i}-free starvation medium (133 mM glucose, 10 mM (NH_{4})_{2}SO_{4}, 13.3 mM KCl, 4 mM CaCl_{2}, 12.5 mM Na_{2} succinate, 1× Verduyn trace elements and vitamins according to Verduyn, Postma, Scheffers, and Van Dijken (1992), and pH 5 with HCl/NaOH) with mild agitation. After cell harvesting and one washing with sterile water, starved S. cerevisiae was stored overnight at 4°C, and incubated at 12°C for 7 days to allow the agglomeration of the insoluble protein and nucleic acid. The agglomerated pellets were removed by centrifugation. The supernatant was centrifuged (10,000 g, 5 min). The pellet was discarded, the volume of the supernatant measured (v_{\gamma} g), and the supernatant moved to a new centrifugation bucket. 8. To precipitate the protein and nucleic acid, 0.02 vol of v_{\gamma} HCl (2.5 M) was added, and the content mixed. The precipitated protein and nucleic acid were removed by centrifugation (10,000 g, 15 min). The centrifuged supernatant was stored overnight at 4°C, and incubated at 7.5 g cell dry weight × L^{-1} and 30°C anaerobically for 2.5 hr in feeding medium (250 mM glucose, 60 mM KH_{2}PO_{4}, 20 mM MgCl_{2}, and pH 6 with HCl/KOH) with mild agitation. PolyP-rich S. cerevisiae was washed twice with sterile water and dried for 5 min on P_{i}-free filter paper to obtain a wet cell mass that contained ca. 25% dry matter. 2. The wet cell mass (weight w_{2}) was transferred to an aluminum flask and stored at -20°C. 3. The cell mass was thawed. 4. To the cell mass, 5 ml autoclaved Milli-Q water was added g wet cell mass (referring to w_{2}) and mixed. 5. The flask was set to 7 with the help of a pH electrode with NaOH or KOH (both 2.5 M) was added and gently swirled over the pellet to remove salt residues from the pellet and bucket walls. 6. The flask was placed on ice for 5 min to cool the content down to room temperature. The content was moved to a centrifugation bucket. 7. The insoluble matter was removed by centrifugation (10,000 g, 5 min). The pellet was discarded, the volume of the supernatant measured (v_{\gamma}), and the supernatant moved to a new centrifugation bucket. 8. To precipitate the protein and nucleic acid, 0.02 vol of v_{\gamma} HCl (2.5 M) was added, and the content mixed. Because polyP hydrolyzes at low pH values, Steps 9–11 were carried out quickly. It was important not to change the order of Steps 8–10 because the HCl precipitation was reversible by alkali addition. 9. The insoluble protein and nucleic acid were removed by centrifugation (10,000 g, 15 min). The prolonged centrifugation time was necessary due to the fine nature of the suspended solids. The supernatant was moved to a new centrifugation bucket, while the pellet was discarded. 10. To neutralize the HCl from Step 8, 0.02 vol of v_{\gamma} NaOH or KOH (both 2.5 M) was added after the centrifugation, and the solution was mixed. 11. The pH was set to 7 with the help of a pH electrode with NaOH or KOH. The used alkali volume was noted. The overall volume (v_{11}) was calculated by adding the volumes of Steps 8 and 10, and to v_{\gamma}, 12. Either (0.020 - 0.010 × v_{\gamma} × [v_{11}]^{-1}) vol of v_{11} NaCl (5 M) or (0.028 - 0.014 × v_{\gamma} × [v_{11}]^{-1}) vol of v_{11} KCl (3.5 M) was added and the solution mixed. 13. To precipitate the intermediate-chain polyP, 0.156 vol of v_{12} ethanol (96% v/v) was added, and the liquid mixed. 14. The suspension was incubated for 1 hr without stirring. It was important not to stir to allow the agglomeration of the polyP. 15. The suspension was centrifuged (10,000 g, 5 min). The pellet and supernatant were separated. The pellet contained the intermediate-chain polyP (ca. 30–40 P-subunits) and was treated according to Steps 19–21. The supernatant, which contained the short-chain polyP (ca. 11 P-subunits), was moved to a new centrifugation bucket and treated according to Steps 16–21. 16. To precipitate the short-chain polyP, 0.885 vol of v_{12} ethanol was added, and the suspension mixed. 17. The suspension was incubated for 1 hr without stirring. 18. The suspension was centrifuged (10,000 g, 10 min). The short-chain polyP was in the pellet. The supernatant was discarded. 19. To each the intermediate-chain and short-chain polyP pellets, 0.5 vol of w_{2} ethanol (50% v/v) was added and gently swirled over the pellet to remove salt residues from the pellet and bucket walls. 20. The polyP pellet remained in the centrifugation bucket. To dry the polyP, the open bucket was placed in a desiccator, which was filled with dry silica, for 1 week. 21. The desiccator-dry polyP was milled by adding ca. 0.5 g polyP and three steel beads (3.2 mm diameter) into a 2 ml stainless steel tube. The steel tube was capped with a silicone rubber plug cap before milling for 2 min in a bead beater. The use of the steel tube and silicone cap was mandatory because other container materials might break during milling. The polyP was stored in a desiccator that was filled with dry silica. When water was added to dry polyP, the tube was immediately vortexed to avoid clumping of the polyP at the bottom of the container. The dissolution of the bio-polyP took 1–2 hr with strong stirring.

2.3 | PolyP analytics

To determine the cellular polyP content and the average polyP chain length in S. cerevisiae, the polyP was extracted from the cells with an analytical polyP extraction (Christ & Blank, 2018). Briefly, S. cerevisiae was suspended in a pH-buffered ethylenediaminetetraacetic acid (EDTA) solution and lyzed with phenol. The lysate was washed with chloroform and then used for further analysis. The total polyP (only linear polyP and no cyclic polyPi), P_{i}, and the average polyP chain length were determined enzymatically (Christ, Willbold, & Blank, 2019). Briefly, P_{i} was assayed colorimetrically after the addition of a P_{i} detection agent, which contained antimycin, molybdate, ascorbate, and sulfuric acid. For total polyP analysis, polyP_{i} was enzymatically hydrolyzed to n P_{i} by S. cerevisiae exopolyphosphatase 1 and S. cerevisiae inorganic pyrophosphatase 1. The released P_{i} was measured colorimetrically. The average polyP chain length was measured as the quotient of the total polyP concentration and the polyP chain concentration. The polyP chain concentration was quantified in an enzyme cascade with the enzymes S. cerevisiae exopolyphosphatase 1 (polyP_{i} → polyP_{2}), ATP sulfurylase (polyP_{2} + AMP-sulfate → ATP + sulfate), hexokinase (ATP + glucose → ADP + glucose 6-phosphate), and glucose 6-phosphate dehydrogenase (glucose 6-phosphate + NADP^{+} → 6-phosphogluconolactone + NADPH, NADPH measured fluorometrically). For the study of the precipitation behavior of chemically produced polyP (Figure 2), the total polyP was measured gravimetrically after drying the dissolved polyP at 120°C. To determine the water solubility and pH of the polyP, a 1% (w/v) polyP suspension was vigorously stirred for 5 hr. If some of the polyP did not dissolve, the suspension was centrifuged (5 min, 10,000 g), the pellet dried in a desiccator for 7 days, and the insoluble matter weighed. The polyP chain length distribution was analyzed with polyacrylamide gel electrophoresis (PAGE; Smith, Wang, & Morrissey, 2018). Briefly,
48 nmol polyP \times \text{lane}^{-1} was separated for 35 min at 150 V and room temperature on a 4–20% gradient polyacrylamide gel (8 cm × 8 cm × 1 mm) in Tris–borate–EDTA buffer. The NEB low range ladder was used as a chain length standard. The corresponding polyP chain lengths were calculated from the DNA chain lengths. The gel was stained with toluidine blue O after electrophoresis. The water content in the polyP was quantified by gravimetric measurement of the water loss at 120°C. Chloride in the polyP was assayed by ion chromatography. The nucleic acid and protein content in the bio-polyP were estimated with a NanoDrop (Thermo Fisher Scientific) by unspecific UV absorption measurement. The polyP purity was determined by measuring the polyP concentration with the Christ et al. (2020) enzyme assay, and quantifying the cation composition with atomic absorption spectroscopy or optical emission spectroscopy (see Supplementary Information for the calculations). The cyclic polyP content was measured with \(^{31}\text{P} \text{NMR}\) (Glonek, Van Wazer, Mudgett, & Myers, 1972; see Supplementary Information for the calculations).

3 | RESULTS

The envisaged workflow for the synthesis of bio-polyP included six process steps. In process Step 1, \textit{S. cerevisiae} was starved in Pi-free medium (P\textsubscript{i} starvation). The starved cell mass was subsequently moved to P\textsubscript{i}-containing medium, where \textit{S. cerevisiae} took up P\textsubscript{i} and intracellularly polymerized it into polyP (P\textsubscript{i} feeding, Step 2). The combination of Steps 1 and 2 is called polyP hyperaccumulation and was already developed in a previous study (Christ & Blank, 2019). In process Step 3, the polyP was liberated from the yeast cell and brought into aqueous solution. In process Step 4, the dissolved polyP was recovered and purified by precipitation. Afterward, the polyP was dried and milled (process Steps 5 and 6). Process Steps 3–6 were developed in this study.

3.1 | Optimal conditions for the precipitation, drying, and milling of polyP

The first aim was to understand which process conditions were required for the precipitation, drying, and milling of polyP. The process conditions that were developed here with chemically produced polyP were later used for the synthesis of bio-polyP. To verify that polyP can be precipitated with an organic solvent, a sodium polyP (Budit 4) was precipitated with 2 vol ethanol and different concentrations of NaCl or KCl. (c) A Budit 4-salt mixture (30 g × L\textsuperscript{-1}, pH to 7 with NaOH or KOH, 100 mM NaCl or KCl) was precipitated with different amounts of ethanol. (d) 730 mg Budit 4 gel (30 g Budit 4 × L\textsuperscript{-1}, pH to 7 with NaOH or KOH, precipitated with 100 mM NaCl or KCl and 2 vol ethanol) was distributed on a 22 cm\textsuperscript{2} drying tray and dried in a desiccator that was filled with dried silica. Mean values from two independent experiments with each one (b–d) or two (a) replicate measurements are shown. Error bars indicate the standard error of the mean between experiments. Abbreviation: w/o, without.
as a food additive, ethanol was chosen as it has the lowest toxicity of the three tested compounds. The different polyP cation compositions were achieved by displacing the counterions with either Na⁺ or K⁺. Budit 4 was precipitated with a combination of different concentrations of NaCl or KCl, and 2 vol ethanol (Figure 2b). At a concentration of 50–750 mM of either salt, Budit 4 was fully recovered (>95%). Because the recovered polyP was measured gravimetrically, it was concluded that NaCl itself did not precipitate. The precipitation with 500 and 750 mM KCl led to the formation of a crystalline precipitate (probably KCl) on top of the polyP sediment. The increasing recovery with 250–750 mM KCl was explained by the precipitation of KCl itself and/or the displacement of the sodium polyP cation into the heavier potassium. A low salt concentration was chosen (100 mM NaCl or KCl). Point-one-five, 0.25, and 0.5 vol ethanol in combination with 100 mM NaCl or KCl were suitable concentrations for fractional polyP precipitation because only ca. 40%, 76%, and 87% of the polyP was recovered, respectively (Figure 2c). One vol ethanol was chosen for full polyP precipitation (92% and 91% recovery with NaCl and KCl, respectively). Drying of the polyP gel was done in a desiccator that was filled with dried silica (without vacuum, Figure 2d). The initial water content of the sodium and potassium polyP gels amounted to 49.0% and 42.6%, respectively. After 7 days, water contents of 0.9% and 1.6% were measured in the sodium and potassium polyP gels, respectively. The water content of unprocessed Budit 4 was 0.2 ± 0.0% (mean ± standard error of the mean (SEM), five replicate measurements). The obtained water content was considered adequate for storage and milling. Budit 4 was recovered as a coarse white crust after drying. To obtain a homogenous fine-grained powder, polyP was milled for 2 min in a bead beater. The fine-grained sodium and potassium polyP powders dissolved readily in water. As for all polyPs, prolonged vigorous stirring was necessary during dissolution to avoid the formation of clumps. The pH of a 1% (w/v) sodium polyP solution amounted to 7.4 ± 0.0, and the pH of the potassium polyP solution was measured at 7.2 ± 0.0 (mean between two independent batches ± SEM). With a pH of 7.0 before the precipitation, this indicated that the pH remained almost unchanged throughout precipitation, drying, and milling.

### Biotechnological synthesis of polyP

The polyP content in polyP-rich *S. cerevisiae* amounted to 26.5 ± 0.8% polyP (as KPO₃) in cell dry weight with an average polyP chain length of 24 ± 1 P-subunits (mean ± SEM from three analytical extractions). The starting protocol for the preparative extraction included a heat treatment to release the polyP from cells, pH neutralization, and precipitation with NaCl-ethanol. The parameters of the heat treatment (1 hr, 70°C) were inspired by Kuroda et al. (2002) who employed those parameters to release polyP from sewage sludge. Two dependent variables (extraction efficiency and average polyP chain length) were analyzed during the optimization experiments. The amount of extracted polyP and the chain length was constant for 3.5–8 ml water per g wet cell mass (Figure 3a). About 5 ml water per g of wet cell mass was chosen. Interestingly, the bio-polyP did not precipitate as a gel but as a solid due to the presence of higher valent cations. An incubation time of 10 min led to the highest extraction efficiency and was, thus, chosen (Figure 3b). The chain length decreased significantly by 1 P-subunit per 10 min due to the heat catalyzed hydrolysis of the polymer (multiple correlation coefficient r = .983, p < .001). No shorter incubation time was tested because quicker heating and cooling would increase the process cost in an upscaling. Because the highest extraction efficiency was found at an incubation temperature of 70°C, this temperature was chosen (Figure 3c). The pH after the heat treatment was acidic. Dilute NaOH was tested as an extractant instead of pure water to immediately neutralize the pH (Figure 3d). About 1 mM NaOH showed the same performance as pure water. Five and 10 mM NaOH decreased the extraction efficiency and the polyP chain length. Fifty and 100 mM NaOH increased the extraction efficiency but decreased the chain length profoundly, and led to precipitation during pH neutralization. Pure water was chosen. Dilute NaCl is commonly used to liberate RNA, which behaves chemically somewhat similar to polyP, from yeast cells (Kuninaka, Fujimoto, Uchida, & Yoshino, 1980). All tested NaCl concentrations (1–200 mM) reduced both the extraction efficiency and the chain length (Figure 3e). To remove protein and nucleic acid, an HCl precipitation was inserted before the ethanol precipitation. PolyP keeps its negative charge, even at very low pH, due to the low pKₐ value (pKₐ = 0–3) of all but two hydroxyl groups per polymer. In contrast, protein and nucleic acid protonate and precipitate at low pH. The ratio of polyP to protein and nucleic acid was increased from 4.1 to 6.2 (w/w), while the extraction efficiency and chain length decreased only by 2.2% points and 0.6 P-subunits, respectively, if 50 mM HCl was used (Figure 3f). Intermediate-chain polyP (41 P-subunits) was recovered in a fractional precipitation with 0.15 vol ethanol (26.9% extraction efficiency, Figure 3g,h). The remaining short-chain polyP (18 P-subunits, 52.5% extraction efficiency) was recovered by adding 1 final vol ethanol. Overall, 80% of the polyP was recovered, which agreed with the extraction efficiency that was obtained with only one precipitation step with 1 vol ethanol.

### Physicochemical characterization of the bio-polyPs

The synthesis of bio-polyP was scaled up by Factor 200 (from 5 mg to 1 g). In the last paragraph, the bio-polyP was liberated from the cells and precipitated with ethanol. The analytics were done with the pellet that was obtained after the ethanol precipitation. In this paragraph, the bio-polyP was dried and milled. A potassium bio-polyP was produced as well as a sodium bio-polyP. The molecular structures of the four newly synthesized bio-polyPs are displayed in Figure 4. The results of the physicochemical characterization of the bio-polyPs in comparison to the three longest, in bulk available commercial polyPs are shown in Table 1. All polyPs appeared as a fine-grained white powder, except for polyP P100, which was delivered as large
FIGURE 3  Liberation of bio-polyP from polyP-rich Saccharomyces cerevisiae and fractional precipitation of bio-polyP. The extraction efficiency was calculated by dividing the amount of recovered polyP by the amount of polyP that was extractable with the reference method [analytical polyP extraction from Christ & Blank (2018)]. (a–h) Show individual experiments that build upon each other. 100 mg wet cell mass (25% dry matter) was suspended in different volumes of water in a 2 ml reaction tube. The suspension was incubated for 1 hr at 70°C and 750 rpm with one 3.2 mm stainless steel bead per reaction tube to facilitate agitation. After the insoluble matter was removed by centrifugation, the supernatant was transferred to a new reaction tube. The pH was neutralized and the polyP precipitated with 100 mM NaCl and 1 vol ethanol. (a) The volume of water, in which the cell mass was suspended, was varied and set to 5 ml per g wet cell mass. (b) The incubation time of the heat treatment was tested and set to 10 min. (c) The temperature during the heat treatment was analyzed and set to 70°C. (d) Different concentrations of NaOH were tested as extractant. Pure water was chosen. (e) Different NaCl concentrations were tested as extractant. Pure water was chosen. (f) Protein and nucleic acid (measured by their absorbance at 280 nm) were precipitated with HCl after the heat treatment. The protocol was continued with pH neutralization and polyP precipitation. Fifty mM HCl was chosen. (g and h) After the recovery of the first polyP fraction with the ethanol volumes depicted on the x-axis, the remaining polyP (fraction 2) was recovered by adding 1 final vol ethanol. Each measuring point in (a) to (g/h) shows the mean value from duplicate preparative extractions. Error bars indicate the standard error of the mean between extractions.
white flakes. The desired powder form is advantageous in regard to shipping cost, storage stability, and handling. The three commercial polyPs contained, as expected, only sodium as the counterion. The sodium bio-polyPs contained approximately equal molar parts of sodium, potassium, and magnesium. This meant that the cells contained as inorganic cations mostly potassium and magnesium. The potassium bio-polyP contained potassium and magnesium in a 1.7:1 and 2.9:1 (Na + K:Mg) molar ratio for the intermediate- and short-chain variants, respectively. This ratio was a good indicator of the speed of dissolution. The polyP purity is reported including and excluding crystal water. The chemically produced polyPs contained only ≤ 0.3% crystal water because chemical synthesis of polyP involves heating the monomer at high temperatures. The bio-polyPs contained 4.6–6.7% crystal water. If one compares the crystal water content of the intermediate- versus short-chain variants, it can be seen that the crystal water content was 1.0% points higher for the sodium and 1.6% points higher for the potassium polyP. These findings confirm both the overall hydrophilicity of polyP and that the hydrophilicity increases with longer chain lengths. Because crystal water was considered as an acceptable component in the polyP, the polyP purity was reported also including the crystal water. The purity (incl. the crystal water) of the chemically produced polyPs measured between 85.8% and 89.2%. The purities of the bio-polyPs (incl. the crystal water) ranged from 83.0% to 91.7%. The bio-polyP purity was, as desired, comparable with chemically produced polyPs. The intermediate-chain sodium bio-polyP was even 2.5% points purer than the purest chemically produced polyP. The average polyP chain length of Budit 4 and the polyP from Roth was ca. 20 P-subunits. The polyP P100 measured 42 P-subunits, which is currently the longest available chain length if one considers the bulk chemical synthesis of solid water-soluble polyP. The average polyP chain length of intermediate-chain sodium bio-polyP was equal to that of P100. The intermediate-chain potassium bio-polyP was shorter (32 P-subunits). The chain lengths of the short-chain sodium and potassium bio-polyPs were around 12 P-subunits, which was equal to the chain length of Budit 7 (a commercially available sodium polyP). Bio-polyP can be prepared in a wide range of polyP chain lengths, enabling its use in many of the established applications. The polyP chain length distribution was analyzed by PAGE (Figure 5). The bio-polyP batches were applied next to each other on the gel and had the same chain length distribution, which indicated—together with the low standard errors of the mean in the other parameters—good reproducibility of the preparative polyP extraction. The lower limit of the chain length distribution could not be determined with PAGE because all samples contained some polyP with a chain length of < 15 P-subunits, which cannot be stained with toluidine blue O. As a consequence, the average polyP chain length appeared longer with PAGE in comparison to the results of the enzyme assay, which included all chain lengths. Although intermediate-chain sodium polyP and polyP P100 had the same average polyP chain length (42 P-subunits), P100 contained a more heterogeneous mixture of chain lengths (longest chain lengths 314 and 700 P-subunits, respectively). Budit 4 and the polyP from Roth showed the same chain length distribution (up to 207 P-subunits). The short-chain bio-polyPs possessed the narrowest chain length distribution (up to 130 P-subunits). The pH of the chemically produced polyPs was in the range of 3.6–5.4, which is typical for chemically produced intermediate-chain polyP. The pH of the bio-polyPs was neutral. The polyP kept the pH, which was set before the ethanol precipitation, throughout the precipitation, drying, milling, and dissolution. The neutral pH is of interest for applications where an intermediate-chain polyP with a neutral pH is required because chemical synthesis can provide only acidic intermediate-chain polyP. The chemically produced polyPs were fully water-soluble and dissolved within a minute in water. The bio-polyPs were also fully water-soluble, except for the potassium intermediate-chain bio-polyP (6.2% insoluble matter). All bio-polyPs required 1–2 hr to dissolve in water because they contained some magnesium. This slow solubility could be a problem for applications where the polyP must dissolve quickly. All tested bio-polyPs contained ≤ 2% cyclic polyP. The chemically produced polyP contained 5–10% cyclic polyP. The results appear plausible because S. cerevisiae can only produce linear polyP. The little amount of cyclic polyP in the bio-polyP was explained by spontaneous formation during the breakdown of linear polyP. The chemical polyP synthesis is uncontrolled so that the formation of cyclic polyP cannot be ruled out. The cyclic polyP content is a parameter to differentiate between bio-polyP and chemically produced polyP. Cyclic polyP is not deleterious in the production of food products but does not contribute to the function of polyP because only linear polyP complexes higher valent cations (an essential property of polyP). Of the chemically produced polyPs, only P100 contained a small amount of P (0.7%). The intermediate-chain bio-polyPs did not contain P, as well. The sodium and potassium short-chain polyPs contained 1.8% and 0.6% P, respectively. The nucleic acid content of the bio-polyPs was estimated spectrophotometrically, a nonspecific method, to be—as desired—low (0.1–1.7%). NaCl or KCl was used to aid the ethanol precipitation. Both did not precipitate, because no chloride was detected in the bio-polyPs. Arsenic, cadmium, calcium, chromium, copper, iron, lead, nickel, and vanadium were neither
detected in the bio-polyPs nor in the chemically produced polyPs, which is vital for their application as food additives. At the same concentration, potassium pulled down more polyP in comparison with sodium during the first ethanol precipitation. This finding was confirmed by the yields of the preparative extraction, which were 15.9% for intermediate-chain sodium bio-polyP and 21.3% for intermediate-chain potassium bio-polyP. The overall yield of the preparative extraction of the sodium bio-polyP was high (80.6%). The yield of the preparative extraction of the potassium bio-polyP was slightly lower (75%).

### 3.4 Mass fluxes in the biosynthesis of polyP

The substrates and products of the Pi starvation and Pi feeding are displayed in Reaction 1 and Reaction 2, respectively. The production of the cell mass, which was required for Reaction 1, and the vitamins and trace elements in the Pi starvation medium were not included in the reactions. The mass balance of the preparative sodium polyP extraction is depicted in Reaction 3. The increase in the water volume stemmed from cell water. For the preparative extraction of

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**TABLE 1** Properties of bio-polyP and chemically produced polyP

| Origin polyP | PolyP name | This study | Commercial |
|--------------|------------|------------|------------|
| PolyP name   |            | Na-polyP | K-polyP | Commercial |
|              |            | Inter-chain | Short-chain | Inter-chain | Short-chain | Budit 4 | Roth | P100 |
| Appearance   |            | Fine-grained white powder | Large white flakes |
| Average molecular formula polymer | Na<sub>9.9</sub> | Na<sub>3.7</sub> | K<sub>15.3</sub> | K<sub>7.8</sub> | Na<sub>19.2</sub> | Na<sub>30.1</sub> | Na<sub>42.0</sub> |
| Mg<sub>10.5</sub> | Mg<sub>2.5</sub> | Mg<sub>9.1</sub> | Mg<sub>2.7</sub> | Mg<sub>9.1</sub> | Mg<sub>8.5</sub> | Mg<sub>9.1</sub> | Mg<sub>9.1</sub> |
| HP<sub>42.3</sub> | HP<sub>11.3</sub> | HP<sub>32.6</sub> | HP<sub>12.7</sub> | HP<sub>32.6</sub> | HP<sub>12.7</sub> | HP<sub>32.6</sub> | HP<sub>32.6</sub> |
| O<sub>127.8</sub> | O<sub>35.0</sub> | O<sub>98.7</sub> | O<sub>37.9</sub> | O<sub>98.7</sub> | O<sub>37.9</sub> | O<sub>98.7</sub> | O<sub>98.7</sub> |
| Average molecular weight polymer (g × mol<sup>−1</sup>) | 4,320 ± 211 | 1,197 ± 21 | 3,410 ± 52 | 1,362 ± 19 | 1,972 | 2,066 | 4,299 |
| Molar ratio Na + K to Mg in the polymer | 2.1 ± 0.1 | 2.9 ± 0.1 | 1.7 ± 0.0 | 2.9 ± 0.1 | n.a. | n.a. | n.a. |
| Chain length distribution (P-subunits) | <15-314 | <15-130 | <15-314 | <15-130 | <15-207 | <15-207 | <15-700 |
| pH of a 1% (w/v) polyP solution | 6.6 ± 0.0 | 6.8 ± 0.0 | 6.4 ± 0.0 | 6.7 ± 0.0 | 3.6 | 5.4 | 5.3 |
| Crystal water (LOQ: < 0.1%)<a> | 6.7 ± 0.3 | 5.7 ± 0.2 | 6.2 ± 0.0 | 4.6 ± 0.1 | 0.2 | n.d. | 0.3 |
| Average chain length (P-subunits) | 42.3 ± 2.2 | 11.3 ± 0.2 | 32.6 ± 0.6 | 12.3 ± 0.2 | 19.2 | 20.1 | 42.0 |
| By enzyme assay | 122 ± 2 | 76 ± 0 | 110 ± 1 | 72 ± 1 | 90 | 85 | 152 |
| By PAGE | 122 ± 2 | 76 ± 0 | 110 ± 1 | 72 ± 1 | 90 | 85 | 152 |
| PolyP purity (% [w/w])<b> | 85.0 ± 1.3 | 79.3 ± 0.1 | 81.8 ± 1.2 | 78.4 ± 0.2 | 89.0 | 87.5 | 85.5 |
| Linear polyP incl. counterions in desiccator-dry substance | 85.0 ± 1.3 | 79.3 ± 0.1 | 81.8 ± 1.2 | 78.4 ± 0.2 | 89.0 | 87.5 | 85.5 |
| Linear polyP incl. counterions and crystal water in desiccator-dry substance | 91.7 ± 1.6 | 85.0 ± 0.2 | 88.0 ± 1.2 | 83.0 ± 0.3 | 89.2 | 87.5 | 85.8 |
| Impurities in the polyP (% [w/w])<c> | 6.2 ± 0.5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Insolubles (LOQ: < 0.01%)<d> | 6.2 ± 0.5 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Chloride (as Cl<sup>−</sup>, LOQ: < 0.75%) | 2.0 ± 0.2 | 0.7 ± 0.0 | 1.5 ± 0.0 | 7.8 | 10.3 | 5.2 |
| Cyclic polyP (as MPO<sub>3</sub>) | 1.8 ± 0.2 | n.d. | 0.6 ± 0.1 | n.d. | 0.7 |
| P<sub>1</sub> (as PO<sub>4</sub><sup>3−</sup>, LOQ: < 0.01%) | 0.2 ± 0.0 | 1.7 ± 0.1 | 0.1 ± 0.0 | 1.6 ± 0.0 | n.d. | n.d. | n.d. |
| Nucleic acid (as RNA, LOQ: < 0.05%) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Arsenic, cadmium, calcium, chromium, copper, iron, lead, nickel, and vanadium | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| (LOQ: < 0.25%) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Yield preparative extraction (% [mole/mole])<e> | 15.9 ± 1.8 | 64.7 ± 2.4 | 21.3 ± 0.8 | 54.5 ± 0.6 | n.a. | n.a. | n.a. |
| Individual for both polyP fractions | 15.9 ± 1.8 | 64.7 ± 2.4 | 21.3 ± 0.8 | 54.5 ± 0.6 | n.a. | n.a. | n.a. |
| Sum of both polyP fractions | 80.6 ± 0.6 | 75.8 ± 0.2 | n.d. | n.d. | n.d. | n.d. | n.d. |

Note: Preparative polyP extractions were done with polyP-rich S. cerevisiae that contained 26.5% polyP (as KPO<sub>3</sub>) in cell dry weight with an average polyP chain length of 24 P-subunits (polyP extracted by analytical polyP extraction, average chain length determined by enzyme assay). For the bio-polyPs, the mean values ± standard error of the mean from two preparative extractions, which were done independently on two separate days, are shown. Abbreviations: Inter., intermediate; LOQ, limit of quantification; n.a., not applicable; n.d., not detectable.

*a*Refers to desiccator-dry polyP.

*b*The value was defined as: (mole polyP recovered after the preparative extraction) × (mole polyP in the polyP-rich cell mass that was used for the preparative extraction)−1.

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[a]: Preparative polyP extractions were done with polyP-rich S. cerevisiae that contained 26.5% polyP (as KPO<sub>3</sub>) in cell dry weight with an average polyP chain length of 24 P-subunits (polyP extracted by analytical polyP extraction, average chain length determined by enzyme assay). For the bio-polyPs, the mean values ± standard error of the mean from two preparative extractions, which were done independently on two separate days, are shown. Abbreviations: Inter., intermediate; LOQ, limit of quantification; n.a., not applicable; n.d., not detectable.

[b]Refers to desiccator-dry polyP.

[c]The value was defined as: (mole polyP recovered after the preparative extraction) × (mole polyP in the polyP-rich cell mass that was used for the preparative extraction)−1.
Preparative polyP extraction: 103.8g ethanol + 0.1L H₂O + 5.4g dry fed S. cerevisiae + 1.3g polyP,

as NaPO₃ + 0.35g NaCl + 0.28g NaOH + 0.21g HCl

→ 103.8g ethanol + 0.1L H₂O (contaminated with NaCl) + 4.4g dry cell debris + 1g polyP (as NaPO₃) + 0.75g NaCl. (3)

Overall: 3.1L H₂O + 103.8g ethanol + 82.5g glucose + 4.9g Na₂Succinate + 3.2g PO₄³⁻ + 3.2g (NH₄)₂SO₄ + 2.4g KCl + 1.5g dry S. cerevisiae + 1.1g CaCl₂ + 1.1g MgCl₂ + 0.35g NaCl + 0.28g NaOH + 0.21g HCl

→ 3L spent broth + 120.2g ethanol + 0.1L H₂O (contaminated with NaCl) + 4.4g dry cell debris + 1g polyP (as NaPO₃) + 0.75g NaCl. (4)

4 | DISCUSSION

A preparative extraction of polyP from polyP-rich S. cerevisiae was developed. In combination with a previously developed process for polyP-rich S. cerevisiae production (Christ & Blank, 2019), pure water-soluble food-grade polyP in powder form was biotechnologically produced. The food-grade status of the bio-polyP was achieved by using GRAS wild-type (non-Genetically Modified Organism) S. cerevisiae and food-grade chemicals. The bio-polyP counterions sodium, potassium, and magnesium are safe for human consumption. Potassium offers an alternative for sodium-limited human diets. Although the magnesium slowed down the dissolution of the bio-polyP in water, it allowed the recovery of the bio-polyP as a solid (instead of a viscous gel with only monovalent cations) during precipitation with ethanol. No toxic elements were detected in the bio-polyP. The linear structure of bio-polyP was confirmed by ³¹P NMR. Examples of bio-polyP use might be in food (e.g., meat products or soft cheese). Furthermore, it can be used for technical applications.

Currently, there is only the chemical route to produce food-grade polyP on an industrial scale. Chemical polyP synthesis (a condensation reaction) is done by heating pure P, at 400–800°C for several hours. The main challenge of chemical polyP synthesis lies in its dependence on pure substrate (P). P is mined from P rock, purified, and imported as phosphoric acid into countries that do not possess P rock reserves. The substrate for chemical polyP synthesis (P) is obtained by pH neutralization of phosphoric acid with NaOH. Problems associated with P rock mining include an uneven global P rock distribution, the limited nature of P rock, environmental destruction and pollution during P rock mining, contamination of P rock with toxic and radioactive elements, and transportation cost (Reta et al., 2018). Strategies for the more efficient use of P and the recycling of P from unused P waste streams must be developed to sustain human life on earth (Ohtake & Tsuneda, 2019).

We developed a green biotechnological process to synthesize pure food-grade polyP with S. cerevisiae. The biotechnological polyP synthesis...
consumes less energy than the chemical synthesis because it is done at \( \leq 30^\circ C \). In comparison with chemical polyP synthesis, \textit{S. cerevisiae} can directly utilize low Pi concentrations (ca. 10–60 mM P) from impure sources. Chemical polyP synthesis cannot be done from such waste streams without extraction and extensive purification of the Pi. In this study, we used pure P, to feed \textit{S. cerevisiae}. Different kinds of waste streams should be tested as P source, prior to study. The primary requirements for our process include that the P, is dissolved, and other dissolved substances do not inhibit \textit{S. cerevisiae} excessively. Food-grade P, waste streams, such as agricultural plant waste (Carraresi, Berg, & Bröring, 2018; Herrmann, Ruff, & Schwaneberg, 2020; Herrmann, Ruff, Infanzon, & Schwaneberg, 2019) and some spent fermentation broths, would allow the production of food-grade bio-polyP. There are many applications of polyP not related to food (e.g., paint, fertilizer, cleaning agents, and flame-retardants). Food-grade P, waste streams, such as industrial wash water and sewage sludge ash, can be used for biotechnological technical-grade polyP production.

Up to this day, there is only one process that deals with biotechnological polyP synthesis (Hirota, Kuroda, Kato, & Ohtake, 2010; Kuroda et al., 2002; Takiguchi, Kuroda, Ohtake, & Tsuneda, 2019). In the Heathphos process, sewage sludge is heat-treated at 70°C for 1 hr. The released polyP is precipitated with Ca\(^{2+}\) and used as fertilizer. The Heathphos polyP cannot be used in food because the product is neither food-grade (P, source: sewage sludge) nor water-soluble (calcium polyP). The water-solubility of polyP is of importance for food applications where polyP can only display its desired physicochemical properties when dissolved. The herein described process bypasses the disadvantages of the Heathphos process by extracting the polyP from \textit{S. cerevisiae} and polyP precipitation with NaCl (or KCl) and ethanol.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Rainer Schnee, Dr. Daniel Buchold, Dr. André Lemke, Dr. Sven Bach, and the Chemische Fabrik Budenheim KG for supplying us with polyP standards, the analysis of inorganic cations, the quantification of chloride, and providing support regarding the analytical and conceptual questions. We thank Dr. Toshikazu Shiba for the kind gift of several polyPs. The authors would like to thank the Research Institute for baker’s yeast for providing us with the \textit{S. cerevisiae} strain VH2.200. J. J. C. and L. M. B. were supported by the Deutsche Bundesstiftung Umwelt (DBU, 33006/01) and S. A. S. and J. H. M. by grant R35 HL135823 from the National Heart Lung and Blood Institute of the NIH.

**CONFLICTS OF INTEREST**

The authors declare that there are no conflicts of interest. The authors filed two patent applications at the German Patent and Trade Mark Office: patent application number 10 2019 131 561.1 with the title “Zusammensetzung, enthaltend getrocknetes Polyphosphat und Verfahren zur Gewinnung von Polyphosphat aus polyphosphat-haltigen Hefezellen dazu” and patent application number 10 2018 130 081.6 with the title “Polyphosphatreiche Hefeextrakte und Herstellverfahren dazu.”

**AUTHOR CONTRIBUTIONS**

J. J. C. conceived and designed the study and performed the majority of experiments. S. A. S. and J. H. M. determined the polyP chain length distribution. S. W. measured the cyclic polyP content. J. J. C. analyzed and interpreted the results and wrote the manuscript. L. M. B. critically revised the manuscript and initiated and coordinated the study.

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How to cite this article: Christ JJ, Smith SA, Willbold S,
Morrissey JH, Blank LM. Biotechnological synthesis of
water-soluble food-grade polyphosphate with Saccharomyces
cerevisiae. Biotechnology and Bioengineering. 2020;117:
2089–2099. https://doi.org/10.1002/bit.27337