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Biotechnological Production and Valorization of Bio-based Polyphosphate Using *Sacharomyces cerevisiae CBS:1502* Strain

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Abstract

Phosphorus, primarily sourced from rock phosphate, is essential for all living organisms and is widely used in agriculture, food, cosmetics, animal feed, and electronics. However, the demand for phosphate exceeds its availability, threatening future supplies. To address this, biotechnologists are investigating methods to recover and recycle phosphate, focusing on extracting polyphosphate from waste streams. This study explores the capacity of the yeast *Saccharomyces cerevisiae* to bioaccumulate free phosphate from a synthetic medium with high phosphate concentrations. Our experiments demonstrated a significant decrease in phosphate levels over time, indicating consumption by the yeast. The strain utilized in our study was able to store a maximum of 10% polyphosphate, which, while lower than the 28% storage capacity reported for other strains, still highlights the potential of yeast to absorb phosphate from environments with elevated levels. This approach not only offers a method for addressing eutrophication in aquatic ecosystems by removing excess phosphates, but also promotes a circular economy. The extracted polyphosphate can be repurposed for applications such as fire control and agriculture, thereby reducing dependence on new phosphate sources and fostering a more sustainable environment.

Keywords: Polyphosphates, *Saccharomyces cerevisiae*, microbial biotechnology, starving medium, feeding medium

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INTRODUCTION

Phosphate is a vital chemical compound that is crucial in various biological processes. It is primarily found in the form of phosphate ions ($PO4^{3-}$). It is an essential component of DNA, RNA, and ATP (adenosine triphosphate), the primary energy carrier in cells (Bruna et al., 2022). Phosphate is involved in energy transfer, signaling pathways, and the structural integrity of cellular components (Michigami et al., 2018; Vulla et al., 2025).

Its presence is critical for maintaining cellular function and metabolic processes, as for growth and reproduction in organisms. Its importance extends to cellular structure, contributing to the formation of phospholipids, vital components of cell membranes. Without sufficient phosphate, organisms may experience stunted growth, impaired metabolism, and reduced fertility (Grünberg, 2014). In yeast cells, phosphate uptake is a well-studied process involving specific transporters in the cell membrane, such as Pho4 as transcription factor, Pho80 as cyclin-CDK complex, and Pho81 as CDK inhibitor active under low phosphate. These transporters facilitate the absorption of inorganic phosphate from the environment, which is critical for the cell's metabolic activities. Yeast cells possess a variety of phosphate transport systems that can adapt to different environmental conditions, allowing them to efficiently utilize available phosphate sources (Soetan et al., 2010; Ibtisham et al., 2018). Research has shown that phosphate uptake is regulated by various factors, including nutrient availability and cellular energy status (Giots et al., 2003;

Smets et al., 2010). This regulation ensures that yeast cells maintain optimal phosphate levels for their metabolic needs Yeast cells store phosphate primarily in the form of polyphosphate granules, which are intracellular structures composed of linear chains of phosphate molecules (Albi & Serrano, 2016). These granules serve as a reservoir of phosphate that can be mobilized when external phosphate levels are low (Xu et al., 2020). Additionally, yeast can also store phosphate in the form of nucleotides and nucleic acids, which are essential for cellular functions (Agledal et al., 2010). The ability to store and utilize phosphate efficiently is crucial for yeast survival, particularly in environments where phosphate is scarce. Phosphate uptake by microorganisms is an important issue in our society due to its implications for agriculture, environmental sustainability, and water quality. In agricultural practices, phosphate is often applied as a fertilizer to enhance crop yields. However, excessive phosphate runoff from agricultural lands can lead to eutrophication in aquatic systems, resulting in harmful algal blooms and degradation of water quality (Sharpley & Wang, 2014; Wurtsbaugh et al., 2019; Badamasi et al., 2019). Furthermore, microorganisms play a critical role in biogeochemical cycles, influencing the availability of phosphate in various ecosystems. Understanding how microorganisms, including yeast, uptake and utilize phosphate can inform sustainable agricultural practices and help mitigate environmental impacts.The present study aimed at studying and optimizing the accumulation of (poly)phosphates by yeast (Saccharomyces cerevisiae). The activities involved the cultivation of yeast, extraction of polyphosphate-rich extracts, and the subsequent analysis of the extract to quantify the total amount of polyphosphate accumulated in the yeast cells.

MATERIALS AND METHODS

Materials

Yeast Strain and Growth Conditions

Yeast cells (*Saccharomyces cerevisiae CBS:1502*) were utilized in the experiment due to their ability to accumulate phosphate from the surrounding environment to synthesize polyphosphates with levels reaching up to 28%. The yeast cells were cultivated anaerobically at a consistent temperature of 28°C across all media employed in the study.

Cultivation media

A total of four different cultivation media were used in this experiment. The composition and preparation of the media were as follows:

YEG media

YEG is a nutrient-rich medium that contains all the essential nutrients required for the growth of yeast cells. It was prepared by mixing 5g of yeast extract and 10g of D- glucose in 1 liter of Milli-Q water. The medium was then sterilized at 121°C for 15 minutes and stored in the refrigerator at 4°C, making it ready for the experiment. The solid YEG media was prepared by mixing 2.5g of yeast extract, 5 g of D-glucose, and 7.5 g of agar in 0.5 L Milli-Q water. The media was also sterilized at 121°C for 15 minutes. After sterilization, 15 ml of the media was poured into 8 sterile test tubes in slanting position and 20 ml in petri dishes. The media was allowed to solidify and stored at 4°C in a cold room.

Yeast Nitrogen Base (YNB) Medium

A 10-times concentrated YNB (without amino acids) solution was prepared by mixing 6.7 g of YNB medium and 20 g of dextrose sugar in 100 ml of Milli-Q water. The mixture was thoroughly mixed, sterile filtered, and stored in the fridge at 4°C. When using the medium for cultivation, the solution is mixed with sterile water in a 1:9 ratio.

Starving medium

This medium contains all the essential nutrients for the growth of yeast (Saccharomyces cerevisiae) except phosphate, to deplete the cells of phosphate. Firstly, 1000-times concentrated vitamin and 100-times trace elements solutions were prepared following the Verduyn et al. (1992) protocol. A specific amount of biotin, calcium pantothenate, nicotinic acid, inositol, thiamine, pyridoxine, and para- aminobenzoic acid to prepare the 1000-times concentrated vitamin solution. Then, a 100-times concentrated trace elements solution were prepared by combining various chemicals. Finally, a mixture of glucose, (NH4)2SO4, KCl, $CaCl_2.2H_2O$, Na2succinate, 1000-times concentrated vitamin solution, and 100-times concentrated trace elements. solution to create the final starvation medium. The pH was adjusted using NaOH and HCl to 5.0, and the medium was sterile-filtered and stored at 4°C.

Feeding medium

To nourish the yeast cells and encourage the accumulation of phosphates, leading to the production of polyphosphates, a two-component medium, referred to as a feeding medium with two components A and B, is used. To prepare 1 L of component A, 50 g of glucose and 11.6 g of K_2HPO_4 were mixed in a 1 L beaker, followed by 800 ml of ultra-pure water. The pH was adjusted to 6.4 using 1M HCl or 1M KOH. The resultant mixture was then transferred to a 1 L graduated flask, brought to a total volume of 1000 ml with Milli-Q water, sterile filtered, and stored at 4°C. Meanwhile, 250 ml of component B was prepared by dissolving 10.175 g of $MgCl_2$ in 250 ml of Milli-Q water. Component B was also sterile filtered and stored at 4°C. Before inoculation, components A and B were mixed in a 9:1 volume ratio to form the complete feeding medium. The two media were mixed immediately before the inoculation to prevent the formation of magnesium phosphate precipitate.

Experimental Procedures

i. Activation phase

Yeast cells, which were previously stored in a deep freezer, were consecutively activated by culturing in YEG broth and agar media. Firstly, a single cell colony was taken using a sterile inoculation loop under sterile conditions and inoculated in 10 ml YEG broth media in 100 ml Erlenmeyer flasks at 28°C for 24 h while agitating.

After 24-h of incubation a plastic spreader was immersed in YEG broth medium and then carefully spread on the surface of the YEG agar medium in petri dishes and test tubes for 48-h.

ii. Adaptation phase

This is a crucial step for adapting the yeast cells to the environment with high phosphate concentration. A single colony was taken from the YEG agar media using an inoculation loop and incubated in 10 ml of YNB media in a 100 ml Erlenmeyer flask at 28°C for 24 h. After 24 h of incubation, 1 ml of the culture medium suspension was taken and placed in a 2 ml sterile Eppendorf tube, centrifuged at 10000 rpm for 5 min, and the supernatant was discarded. The cells were washed twice with 1 ml of sterile Milli-Q to clean all the YEG media components.

A new medium mix was made by mixing 9 ml of sterile distilled water and 1 ml of 10X concentrated YNB in a sterile 100 ml Erlenmeyer flask. 1 ml of sterile Milli-Q water was then added to the washed cell pellet in the Eppendorf tube and thoroughly mixed. Sterile Milli-Q water was then added to the washed cell pellet in the Eppendorf tube. The suspension was then added to the 10 ml freshly prepared YNB medium and incubated at 28°C for 24-h while agitating. After 24-h, all 10 ml of the YNB was taken, added into 90 ml of YNB in 1-L flask, and incubated at 28°C for 24- h.

iii. Starving phase

This is a step when the yeast cells are placed in a medium with all essential nutrients for their growth, except the phosphate. After 24 h of incubation in the YNB medium, the cell suspension was centrifuged at 4500 rpm for 10 min, and the supernatant was discarded. The pellet was washed with sterile Milli-Q water and centrifuged at 4500 rpm for 10 minutes. The cell pellet was resuspended with sterile Milli-Q water, and the optical density (OD600) and the volume of the suspension were determined, which should added to the starving media. Equation (3) helped determine the volume of cell suspension to be added to the starving media. The volume of the cell suspension taken should correspond to 0.625 g of cell (dry weight). The determined cell suspension volume was then centrifuged at 4500 rpm for 5 minutes; the cell pellet was taken and transferred to 100 ml of starving media in a 1-L Erlenmeyer flask and incubated at 28°C for 6 h. After 6-h of starving the yeasts, the medium suspension was centrifuged at 4500 rpm for 10 min, and the supernatant was discarded. The pellet was then washed with sterile distilled water by centrifugation and the cell mass was stored at 4°C for 17-h. The 17-h of storage are unnecessary; you can go to the feeding step instantly if time allows.

$$V\ cell\ suspension\ =\ Qcell/\ OD600 * k \qquad (1)$$

Where as;

Qcell: Quantinty of cells in grams (dry weight basis)
V cell suspension: Volume of cells suspension to the taken
OD600: Optical density of cells suspension at 600 nm
K: Coefficient factor showing the relation between OD600 and concentration of cells

iv. Feeding phase

This is the step when the yeast cells are now fed with the media containing a known concentration of phosphate. The starved cells were resuspended with Milli-Q water and the OD600 was determined. Thanks to equation (3). The same equation was used to determine the volume of the cell suspension that should be added to the feeding media. The determined volume was then centrifuged and the cell pellet was obtained.

a Added to 10 ml of feeding media. The volume of the cell suspension should correspond to 0.75 g (dry mass). The inoculated feeding media were incubated at 28°C for 2.5 h. After 2.5 h, the cell suspension was centrifuged at 4500 rpm for 10 min, and the supernatant was discarded. The cells were washed with sterile milli-Q water and centrifuged to obtain the cell pellet ready for extraction and quantification of polyphosphate.

Growth monitoring

During the adaptation period, the growth of the yeast was monitored by taking samples every two hours for 24 hours and measuring the optical density (OD600) using the JEENWAY 7315 spectrophotometer (Cole-Parmer Ltd, Staffordshire, United Kingdom) and the microscopic cell counts for the cell concentration. This was done to determine when the yeast stopped growing and reached the plateau.

Determination of Cell Dry Mass

The cell dry mass was determined by taking a specified amount of fresh yeast cells (at the beginning of the experiment) or a specific volume of medium suspension (at the end), centrifuging with pure water and obtaining the cell pellet. The fresh cells or cell pellets were then resuspended with a known volume of water and placed in a preweighed beaker. The cell suspension in beakers was oven-dried at 103 °C and for 24 h. After 24 h, the beakers were reweighed and the dry mass was calculated per equation 2.

Weight of beaker + dried sample(W2) - Weight of empty beaker (W1) (2)

Enzymatic colorimetric determination of glucose in the media

Samples of the media were collected during feeding from time zero and after every 15 min until 150 min. Glucose was determined by colorimetric method through glucose oxidation by glucose peroxidase to produce hydrogen peroxide and gluconic acid. The hydrogen peroxide then reacts with aminophenazone and phenol to form a red compound measured at 510 nm. Glucose was then quantified as per equation 3.

Glucose (mg/dl) = (absorbance of sample * 100 Absorbance of standard) (3)

Determination of Free-phosphate (Pi) in the Medium

The disappearance of free phosphate in the medium during the feeding stage was determined by the molybdenum blue method. Samples of the medium were collected at regular intervals from time zero and after every 15 minutes until 150 minutes, when the feeding stopped. Samples were centrifuged and all the cells were removed, and the free-phosphate concentration was determined in the remaining supernatant after dilution. The concentration of the free phosphate was then quantified using the calibration curve.

Total polyphosphate quantification

The total polyphosphate accumulated in yeast cells was quantified using an Aminoverse Total Polyphosphate Quantification Kit developed by Christ and Blank (2018). The Kit can quantify even contaminated samples down to two polyphosphate chains. Phosphate detection reagent and enzyme master mix were prepared according to the manufacturer's instructions. Samples were diluted to laboratory-grade water between 5 and 200 μM polyphosphate as monomer. Negative control, 100 μL and 100 μL of the Pi standards were added to individual wells on a microtiter plate. 100 μL of the sample under different dilutions was added into different wells, too. Positive control was also added into another well. Each sample and positive control requires two wells, of which one is for the actual measurement and another for the blank measurement that detects contaminating substances. 50 μL of enzyme reaction buffer was added to the Pi standards, one positive control, and the blank measurements. 50 μL of enzyme master mix was added to the negative control, the other positive control, and the samples. All contents in wells were thoroughly mixed by pipetting three times up and down and incubated at room temperature for 1-h. After 1-h of incubation, 50 μL of the phosphate detection reagent was added to every well, mixed by pipetting three times up and down, and incubated at room temperature for 2 min. The absorbance was then read at 882 nm wavelength in a spectrophotometric plate reader without the plate lid, and the sample polyP concentration was calculated using the Pi calibration curve.

Microscopic observation of polyphosphate granules in yeast cells

This short and straightforward procedure is necessary for viewing the accumulated polyP in yeast cells. At the end of the experiment, a staining mixture comprised of glacial acetic acid 1.5 ml, saturated aqueous toluidine blue O 4 ml, and 20 ml of formalin was made for viewing polyphosphate granules inside the cells as per the previous method by Lindegren (1947). A drop of well-mixed, diluted cell suspension was placed on a microscope slide, followed by an equal amount of staining mixture on the same slide. Volutin was then observed in the yeast cells as a metaphosphate granule.

RESULTS AND DISCUSSION

Results Growth curve

Figure 1 presents the results of the yeast's growth during the adaptation stage in YNB media. It shows that cell growth started after 2 h of incubation, and the growth was exponential until after 16 h, when it reached a plateau, and no further growth was observed.

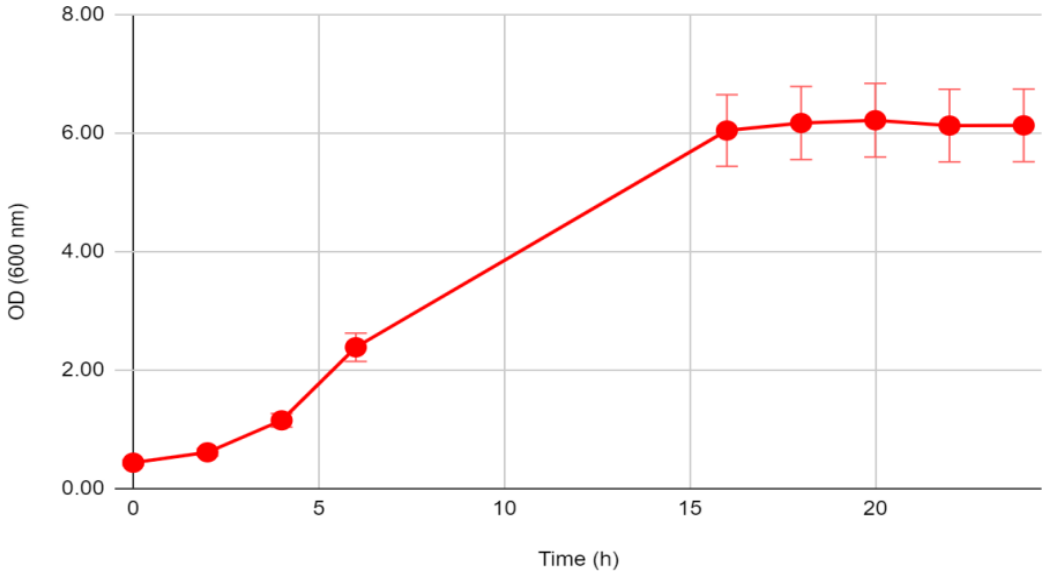

Figure 1. The growth curve of *Saccharomyces cerevisiae* in YNB media

Consumption of glucose in the feeding media

The glucose determination results during the feeding stage indicate a decreasing glucose concentration over time, as illustrated in Table 1 and Figure 2. The total feeding time was 150 minutes, starting with a glucose concentration of 50 g/l. By the end of the experiment, the yeast (7.5 dry weight) had consumed nearly all the glucose reserves, resulting in a concentration of 1.8 g/l. This suggests that the initial glucose concentration for 7.5 g dry weight of yeast during the feeding stage was sufficient, and glucose did not act as a limiting factor. On the other hand for the experiment started with 25 g of fresh yeast directly to the feeding, the results showed that yeast started with 47.8 g/l of glucose and ended up with 11.8 g/l of glucose as shown in Table 1 and Figure 2

Table 1. The concentration of glucose (g/L) during the feeding phase for starved and fresh yeast cells

Time/ Glucose (g/l)	0	15	30	45	60	75	90	105	120	135	150
Series 1 (Starved)	46.6	39.8	38.1	35.5	25.2	22.3	16.6	13.4	7.6	4.1	1.8
Series 2 (fresh)	47.8	47.7	42.7	40.9	37.7	36.0	28.5	23.3	20.2	16.4	11.8

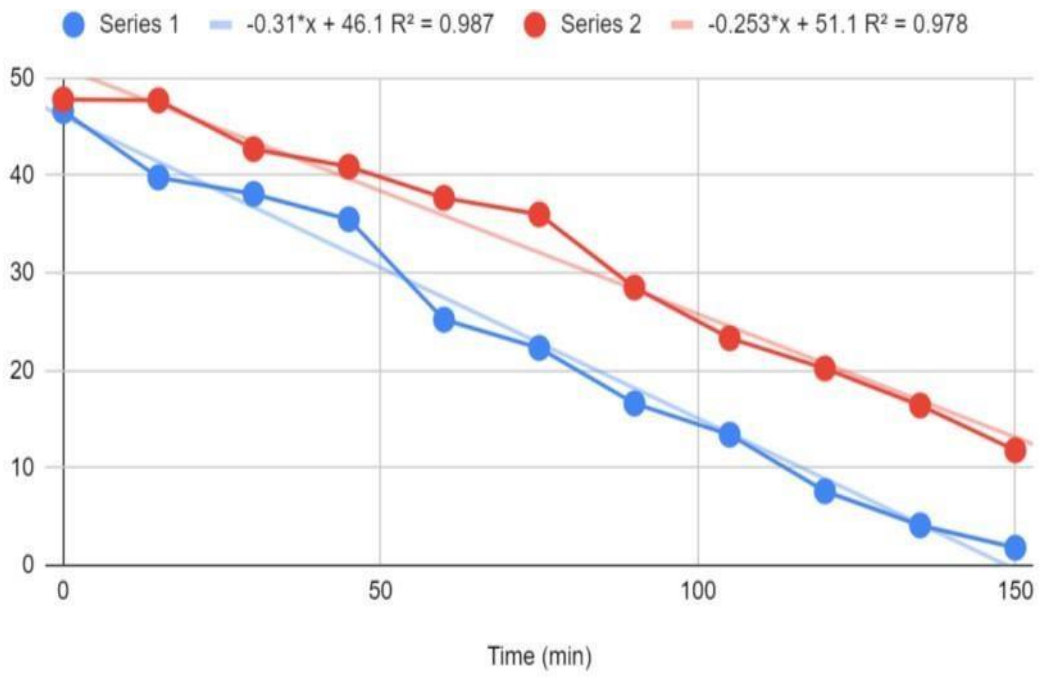

Figure 2. The consumption of glucose overtime during the feeding phase for starved and fresh yeast cells

Disappearance of free phosphate (Pi) in the feeding media

The free phosphate determination results during the feeding stage indicate a decreasing concentration of free phosphate in the media over time, as illustrated in Table 2. The total feeding time was 150 minutes, starting with a glucose concentration of 8.66 and 8.63 g/l for series 1 and 2 respectively. By the end of the experiment, the yeast had consumed only 2.39 and 1.42 g/L of free phosphate. This suggests that the starving stage is crucial for yeast to absorb more phosphate from the feeding medium because starved yeasts have absorbed more phosphate than non-starved yeasts.

Table 2. The concentration of free phosphate (g/L) during the feeding phase

Time/ Glucose (g/l)	0	15	30	45	60	75	90	105	120	135	150
Series 1 (Starved)	8.66	8.38	8.35	8.04	7.54	7.62	7.21	6.59	6.89	6.51	627
Series 2 (fresh)	8.63	8.62	7.66	8.03	8.49	8.41	8.28	8.13	8.07	7.79	721

Results of dry mass determination

The results of the dry mass determination are presented in **Table 3** below. The results showed that the yeast had 29 to 31% dry mass weight.

Table 3. Percentage dry mass of bakers' yeast

S/N	Weight of an empty beaker in g (g)	Weight of beaker + cell suspension (g)	Dry mass	Total	dry mass (%)
1st series					
B1	78.477	79.266	0.796	1.563	31
B2	75.878	76.645	0.767		
2nd series					
B1	78.465	79.186	0.721	1.436	29
B2	75.864	76.579	0.715		

Total polyphosphate quantification in yeast cells

The results of total polyphosphate quantification for different experiments conducted were presented in Table 4.

Table 4. Results of polyphosphate quantification for different series of experiments

Series	Mass of cells (mg)	PolyP (mg)	% PolyP
1	6.25	0.569	9.11
2	6.25	0.645	10.32

Microscopic observation of polyphosphate

After staining the yeast cells with the staining mixture, we observed polyphosphate granules inside the cells, as shown in Figure 7. With this method, polyphosphate inside the cells was observed as metaphosphate in volutin, a cytological entity that has long been recognized as an important constituent of yeast cells. Volutin granules are chiefly metaphosphate and contain calcium phosphate in combination with RN

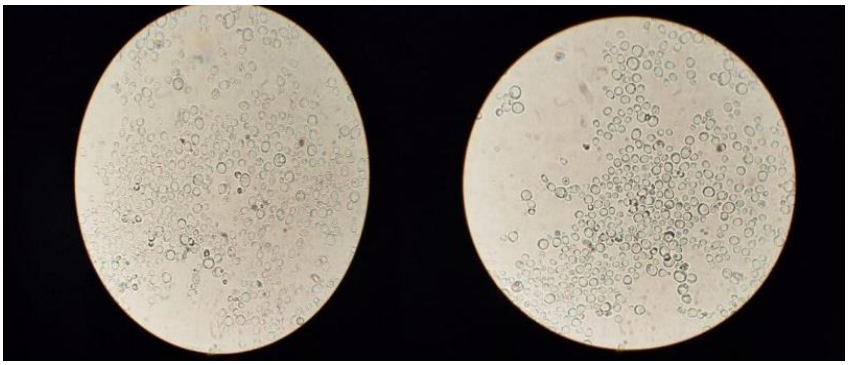

Figure 3. Stained yeast cells with polyphosphate granules inside the cells

DISCUSSION

The yeast reached a plateau phase after 16 hours of incubation in YNB media, indicating that all nutrients in the media had been consumed and the cells could no longer increase in number. However, during starvation, the cells continued to multiply by utilizing the phosphate reserve within the cells. It was observed that the cell concentration doubled during the 6-hour incubation period in phosphate-deficient media. In addition to the absence of phosphate in the media, glucose, vitamins, and mineral solutions were present to provide energy and other essential nutrients for cellular metabolism. This increase in cell concentration is economically beneficial as it results in more cells, which can be advantageous for subsequent feeding stages, leading to increased phosphate into polyphosphate. Research by Christ and Blank demonstrated a decrease in polyphosphate levels from 2.5% in normal cells to 0.1% in starved cells after 2.5 hours. Adding $MgCl_2$ in the feeding media is crucial, as the omission of it can significantly decrease polyphosphate accumulation. High levels of Mg^{2+} are necessary for efficient hyper accumulation of polyphosphate in *Saccharomyces cerevisiae*, as noted by Weinberg (1975).

Feeding the yeast for 2.5 hours is essential, as observed by Christ and Blank (2018), where the polyphosphate content in starved cells increased by 250-fold in just 2.5 hours. This elevated polyphosphate concentration during the feeding stage is 10 times higher than the normal polyphosphate levels in regular yeast cells. Prolonged starvation for more than 2.5 hours resulted in a slight decrease in total polyphosphate content in the cells, underscoring the importance of limiting the starvation period to 2.5 hours. Moreover, during the feeding phase, there was a continual decrease in both the glucose and free phosphate concentrations in the media. This was attributed to the fact that the yeast cells utilized glucose as an energy source to facilitate the synthesis and storage of polyphosphate within the cells. The conversion of free phosphate into polyphosphate is an anabolic process, requiring the yeast to derive energy from the carbon source to build the polyphosphate chain. We had anticipated to extract between 24 and 28% of polyP (quantified as KPO_3) accumulated in yeast cells using the optimized protocol by Christ and Blank (2018). However, we only extracted a maximum of 10.32% despite observing metapolyphosphate under a microscope. We hypothesized that the low yield could be attributed to the yeast strain used, as different strains have varying accumulation capacities. Additionally, we noticed that the extraction process may have been ineffective, as we observed unruptured cells under the microscope, suggesting that not all cells released their accumulated poly-phosphate content.

CONCLUSION

In conclusion, phosphate bio-recycling is a vital strategy for recovering and reusing valuable phosphate resources, significantly reducing our dependence on finite phosphate rock reserves. By harnessing the capabilities of microorganisms, we can effectively recover excess phosphate from various sources, including agri-food waste, and repurpose it across multiple industrial applications. However, further optimization and research are imperative to achieve the scalability and effectiveness necessary for widespread adoption, particularly in industries producing phosphate-rich wastewater.

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