Oxidative stability of monoethanolamine in CO_2 capture using dissolved oxygen measurements

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Full length article

Recovery of C, N, and P from waste activated sludge by enzymatic anaerobic fermentation: Stoichiometry and metatranscriptomics analysis

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ABSTRACT

The recovery of C, N, and P elements by sludge biorefinery potentially reduces operation costs and increases the extra benefits. Herein, we analyzed the elemental stoichiometry of C, N, and P and functional microbiome involved in enzymatic anaerobic fermentation. Enzymatic hydrolysis was observed to increase the release of C, N, and P into the sludge supernatants by 21.8 %–26.3 %. Metatranscriptome analysis indicated that enzymatic pretreatment enhanced the metabolism of the organic carbon degradation, ammonium conversion, and P solubilization in subsequent fermentation. Specifically, enzymatic pretreatment enhanced endogenous carbon hydrolase activity by 48.4 %–72.7 % and upregulated intra-C metabolic pathways, such as glycolysis and pyruvate metabolism. Ammonium transport and conversion were significantly increased by 4–6 fold, stimulating the synthesis of glutamine and endogenous amino acids. Additionally, enzymatic hydrolysis promoted phosphatase secretion and enhanced bacterial P uptake. These effects improved the recovery of C, N, and P as dentification carbon source and struvite by 13.7 %–41.8 % and the dry sludge production was reduced by 24.3 %–28.1 %. Life cycle assessment (LCA) indicated the shift of CO_2 emissions from net positive to net negative levels as compared to the conventional A^2/O process. This study offers valuable insights into the redistribution and metabolism of various elements involved in the enzymatic anaerobic fermentation, and proposes the potential strategy to recovery C, N, and P from sewage via sludge biorefinery.

1. Introduction

In wastewater treatment plants (WWTPs), the generation of substantial waste-activated sludge (WAS) has been viewed as a significant problem, and currently the resource-rich features of WAS are acknowledged by a green-oriented and zero-pollution society (Faragò et al., 2022; Munir et al., 2018; Zeng et al., 2022). WAS has been recognized as important source materials to produce renewable energy, e.g., hydrogen, methane, and biodiesel (Fang et al., 2020; Kwon et al., 2012; Manara and Zabaniotou, 2012), valuable chemicals such as polyhydroxyalkanoate (PHA) (Frison et al., 2015; Pei et al., 2022), single-cell protein (SCPs) (Gu et al., 2024; Wu et al., 2021), and phosphate (Ribarova et al., 2017; Shashvatt et al., 2022), Concurrently, the United Nations' Sustainable Development Goals (SDGs) emphasize comprehensive and integrated resource recovery to enhance global resource cycling and to achieve sustainable development (United Nations, 2015; Trimmer et al., 2019).

Sludge biorefineries have been proposed to advance the recovery of multiple valuable resources, rather than focusing on single elements (Crutchik et al., 2018; Fang et al., 2020; Zhou et al., 2019). For example, WAS fermentation has been reported to achieve the simultaneous recovery of ammonia nitrogen (NH_4^+-N) and volatile fatty acids (VFAs) (Zhang and Chen, 2009). Ethylenediaminetetraacetic acid-enhanced anaerobic digestion has been reported to not only improve sludge methanogenesis but also mobilize up to 80.4 % of the total phosphorus (P), thereby enabling dual resource recovery (Zou et al., 2025b). Compared with conventional biorefinery schemes, integrated multi-resource recovery strategies could greatly improve the potential

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and expand the application scope of resource utilization (Luo et al., 2024; Messa et al., 2025), and this perspective offers significant potential to integrate carbon reduction with holistic valorization in WWTPs within the framework of sustainable development. To the best of our knowledge, despite the recognition of this perspective, research on integrated multi-resource recovery from sludge is relatively scarce and faces the problems of limited recovery efficiency and the high application barrier that erode net benefit. Developing practical and highly efficient recovery strategies is a key area that warrants further investigation.

Recently, various strategies have been explored to enhance sludge biorefinery, including thermal, alkaline, ultrasonic, Fenton, and combined pretreatments (Elalami et al., 2019; Kor-Bicakci and Eskicioglu, 2019). However, their large-scale application is often constrained by the high energy and chemical costs, undesirable side reactions, secondary pollution, and substantial alterations to sludge chemical characteristics, which might hinder downstream bioconversion and the extraction of various resources (Balasundaram et al., 2022; Gonzalez et al., 2018). In contrast, enzymatic pretreatment has been reported to mildly condition sludge and enhance anaerobic fermentation with minimal energy requirements, owing to its superior catalytic efficiency (Odnell et al., 2016; Zou et al., 2025a). It was reported that lysozyme efficiently disrupted the sludge cells and enhanced carbon recovery by 538 %–621 % (Pang et al., 2024). The immobilized hydrolases and laccase was observed to improve the growth of key anaerobes involved in VFAs production (Wan et al., 2022). As polysaccharides and proteins constitute over 60 % of the dry sludge (Neyens and Baeyens, 2003), amylases and proteases have also been investigated for optimizing sludge conditioning and biorefinery. For example, protease has been reported to significantly enhance organic carbon release in sludge and increase methane yield by 86.1 % (Jiang et al., 2024). In our previous study, hydrolysis with amylase and protease improved the sludge solid–liquid interface, thereby facilitating the growth and enrichment of fermentative microorganisms (Song et al., 2024). Compared with other enzymes, proteases and amylases could be produced through biomass conversion to offer lower cost-effectiveness (Contesini et al., 2018; Diamantopoulou et al., 2025), and this further expands their application potential in sludge treatment. Typically, enzymatic hydrolysis serves as a gating reaction that could drive multi-elements cycling and release within a microecosystem (Han et al., 2024; Zhao et al., 2024). This effect is expected to further affect the recovery potential of resource elements such as carbon (C), nitrogen (N), and P in sludge, and their fates during anaerobic fermentation. Unfortunately, the effects of enzymatic anaerobic fermentation on the redistribution and co-recovery of these key elements in sludge are far to be well illustrated, and this restricts the optimization of enzymatic anaerobic fermentation for enhancing environmental benefits with regard to multi-resource recovery and CO_2 emission reduction.

Based on these considerations, this study proposes the enzymatic anaerobic fermentation process to recovery C, N, and P from WAS, and aims to: (1) quantify their redistribution behaviors upon enzymatic pretreatment and anaerobic fermentation by elemental stoichiometric analysis; (2) elucidate the functional microbial communities and metabolic pathways involved in fermentation by metatranscriptomic sequencing and metabolic reconstruction; (3) illustrate the recovery potential of C, N, and P in terms of dentification carbon source and struvite, and evaluate the environmental benefits of sludge biorefinery by life cycle assessment (LCA). This study provides insights into the redistribution and cycling of C, N, and P elements involved in enzymatic anaerobic fermentation, and potentially advances the integrated recovery of multiple valuable resources from WAS.

2. Material and methods

2.1. Sludge samples and enzymatic anaerobic fermentation experiments

The WAS and inoculum used in this study were collected from a secondary sedimentation tank and the mesophilic anaerobic digester at a WWTP in Beijing, China. Their specific characteristics are provided in Table S1 and Text S1. Detailed information regarding the enzymatic pretreatment processes and subsequent acidogenic fermentation experiments were described in our previous paper (Song et al., 2024), and are summarized in Text S2. Protease and amylase were added to the sludge at dosages of 0.0054 g/g TS and 0.027 g/g TS, respectively, to attain the enzymatic activities of 400 U/g VS in the systems. The intrinsic elements and chemical oxygen demand (COD) of the enzymes had insignificant effect on sludge composition, and detailed contributions of the enzymes are provided in Table S2 and Text S3. Additionally, a control pretreatment was established to isolate the effects of shaking and heating on sludge properties from the subsequent fermentation. The groups with protease, α-amylase, and control pretreatment were named Pro, Amy, and Con, and the raw sludge was named RS.

2.2. Sludge component extraction

Based on previous reports (Snidaro et al., 1997; Yu et al., 2008), the stratification structure of sludge was divided into supernatant, extracellular polymeric substances (EPS), intracellular species, and sludge residue. First, the supernatant was obtained after allowing the sludge to settle for 1.5 h at 4 °C (Yu et al., 2008). Then, a modified heating method was used to extract EPS from the sludge (Procházka et al., 2012). The remaining sludge pellet was re-suspended in a 0.75 mol/L LiCl solution. The cells were crushed using an ultrasonic cell crusher (BioSafer-650E, China) at 10 W/mL for 15 min, followed by centrifugation at 20,000 × *g* and 4 °C for 15 min to obtain intracellular biomolecules (Hausmann et al., 2016). The final precipitate retained in the tube was collected as the sludge residue. The detailed protocol is provided in Text S4.

2.3. Resources recovery experiments and life cycle assessment

First, the pH of the fermented sludge was adjusted from 5.5 ± 0.1, 5.3 ± 0.2 and 5.1 ± 0.2 for the Con, Pro and Amy, respectively, to 9.5 using a 0.5 mol/L NaOH solution. Subsequently, a 1.0 mol/L $MgCl_2$ solution was added to adjust the Mg/P molar ratio to 1.2:1.0 in the sludge. The reaction was conducted for 20 min at 130 rpm stirring and ambient temperature, and then the sludge was allowed to settle and age for 2 h (Zeng et al., 2018). The precipitates were filtered and collected using 0.45 μm filters, and the precipitated phosphorus was determined by the difference between the initial and final total phosphorus (TP) concentrations in the sludge. Subsequently, the supernatants from the fermented sludge were collected, and their carbon source concentrations were determined by the soluble COD (SCOD). Based on previous report (Bian et al., 2022), denitrification experiments were conducted in sealed 100 mL glass serum bottles to investigate the bioavailability of the sludge-derived carbon source. Denitrification sludge and *Paracoccus denitrificans* were used as inoculants, and the commercial acetate was used as the control. The effective carbon source in denitrification systems was calculated based on previous report (Zhou et al., 2023). The detailed protocol of denitrification experiment is provided in Text S5.

A life cycle impact assessment (LCIA) model was conducted using the CML 2001 method in GaBi 9.0 (Professional Edition) to compare the environmental impacts of conventional and enzymatic anaerobic fermentation (Figure S1). This study mainly focused on nine categories of midpoint impacts: acidification potential (AP), eutrophication potential (EP), global warming potential (GWP), ozone layer depletion potential (ODP), photochem, ozone creation potential (POCP), terrestric ecotoxicity potential (TETP), freshwater aquatic ecotoxicity potential (FAETP), marine aquatic ecotoxicity potential (MAETP), and human

toxicity potential (HTP) (Zhao et al., 2023a, 2023b). The detailed inventory and additional information of LCA are provided in Text S6. Furthermore, the economic cost analysis was conducted based on previous studies (Yuan et al., 2024). The detailed information are provided in Text S7.

2.4. Analytical methods

The C, N, and S contents in the sludge components were determined using an elemental analyzer (Vario Macro Cube, Elementar, Germany) with a detection limit of 0.01 %. The VFA content (i.e., the sum of acetate, propionate, butyrate, and valerate) was detected using a Shimadzu GC-2010 Plus (Shimadzu, Japan) instrument equipped with a hydrogen flame detector. According to previous reports (He et al., 2012), the released H_2S was collected by absorption in a cadmium hydroxide–ammonium alcohol polyvinyl phosphate solution and measured using the methylene blue spectrophotometric method. The P contents in sludge components and COD were determined using standard methods (APHA, 2017). The life time of exogenous hydrolase was reported to be limited to <24 h in sludge (Odnell et al., 2016; Yang et al., 2010), thus the samples were collected for hydrolase extraction and activity analysis on Day 2 to block the effects of the exogenous enzyme. The extraction of attached and free hydrolase followed previously reported methods (Guo et al., 2021), and the detailed process is provided in Text S8. The activities of amylase and protease were individually analyzed using an Amylase Activity Assay Kit (Solarbio, China) and a Neutral Proteinase Activity Assay Kit (Solarbio, China) with a multimode microplate reader (Spark, Tecan, Switzerland). The hydrolase activity measurements for both methods were performed at 37°C. The concentrations of NO_3^--N were determined using the chromatography of ions (ICS-2000, DIONEX, USA). Optical density at 600 nm (OD_{600}) was determined using the multimode microplate reader (Spark, Tecan, Switzerland).

2.5. RNA extraction, transcriptome sequencing, and metatranscriptome analysis

To elucidate the expression levels of genes involved in element metabolism, sludge samples for the fermentation prophase (Day 1), metaphase (Day 4), and anaphase (Day 15) were collected and stored at −80 °C until use. RNA extraction was performed using the E.Z.N.A.® Soil RNA Midi Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's protocol. The procedures for RNA extraction, library construction, transgenomic sequencing, and genome assembly are detailed in Text S9. Afterward, gene taxonomy, functional annotation, and metabolic pathway analysis were conducted according to previously reported methods, and the detailed procedure is provided in Text S10. Notably, the abundance of genes in each sample was quantified using reads per kilobase per million mapped reads (RPKM) (Kojima Conner et al., 2022).

2.6. Data analyses

All tests in this study were conducted in triplicate, and the values are presented as means ± standard deviation. Data processing was conducted using OriginPro 2023b and R studio (R studio version 1.4.1106, R version 4.0.3). The student's *t*-test was used to assess the significance of the results, with $p < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Distribution and stoichiometry of C, N, and P in enzymatic fermentation

To investigate the effects of enzymatic pretreatment and fermentation on resources redistribution in the sludge, the stoichiometric analysis of C, N, and P was conducted for different sludge fractions and enzymatic hydrolysis was observed to greatly affect their redistribution behaviors (Fig. 1& Table S3). Specifically, the C proportions in the supernatants increased significantly from 1.0 % in RS to 23.4 % and 10.2 % in the Amy and Pro groups, and that in the Con group was as low as 1.6 %. Protease and amylase pretreatment also contributed to significant N release of 12.0 % and 27.8 % into the supernatant, and this was accompanied by the reduced N proportions in EPS and sludge residue. In Pro group, this may be attributed to the unlocking effect of protease towards the nitrogen-rich organics such as amino acids and proteins in Pro group (Guérard et al., 2002). In Amy group, although amylases had limited capacity to hydrolyze nitrogen-rich organics into small molecules, its hydrolysis of polysaccharides reduced EPS structural complexity and the interlinking between proteins and polysaccharides (Basuvaraj et al., 2015). This effect might promote the N solubilization and release in the Amy group. In practical applications, amylase might be more effective in rapidly releasing and co-recovering the C and N resources from sludge during pretreatment. Interestingly, P redistribution showed insignificant difference among different sludge samples, and enzyme hydrolysis can hardly improve P release. Orthophosphate, polyphosphate and organophosphates, such as phospholipids and DNA, were reported to be the predominant forms of P within intracellular components and EPS of sludge (Cassidy and Belia, 2005; Ding et al., 2022). This insignificant P release may be ascribed to the stabilization of organophosphates compounds and phosphates species during enzymatic hydrolysis (Saktaywin et al., 2005).

In the subsequent acidogenic fermentation, the introduction of exogenous hydrolase greatly promoted C dissolution and intracellular C (intra-C) accumulation and decreased CO_2 release accordingly. Nevertheless, the C contents as VFAs were determined to be in the range of 21.4 %–26.2 % in different scenarios, and this was relatively lower than the total C content in the supernatants, i.e., 29.3 %–43.0 %. Additionally, the N proportions in residues of all sludge samples were above 43.0 %, and this was indicative of the insufficient degradation and transformation of macromolecules with refractory organic nitrogen. Amylase hydrolysis slightly increased the N content by 2.9 % in the fermentation liquid, and the proportion of intracellular N (intra-N) increased to 29.4 % and was 1.9-fold higher than that of 15.1 % in the Con group. By contrast, protease significantly increased the N content in the supernatant from 20.6 % in the Con group to 39.8 %. Although protease released less N content than amylase during pretreatment, the micromolecular peptides and amino acids generated from hydrolysis may further enhance N transformation and release during fermentation. In practical applications, protease-enhanced anaerobic fermentation may offer greater potential for the targeted co-recovery of C and N, particularly for producing single-cell proteins and amino acids. Furthermore, 62.6 %–67.1 % of P remained in the sludge residues upon enzymatic pretreatment, and the further fermentation slightly increased the ratios to 73.7 %–81.1 %. Enzymatic pretreatment slightly favored P migration into the supernatant as compared to the conventional fermentation. The high contents of residue P may be attributed to the formation of chemical P precipitates involved in the P removal by Al salts in the mainstream processes (Text S1), and the persistent P resources in sludge residues was also reported in other WWTPs (Liu et al., 2019; Yao et al., 2024). Enzyme introduction also affected the sulfur (S) elements redistribution and H_2S release was observed to be decreased by 37.5 % (Table S3, Figure S2, Figure S3 & Supplementary Results and Discussion). Overall, enzymatic hydrolysis was observed to greatly promote the dissolution of C and N into the supernatants, and this may affect their metabolism and the fermentation behaviors thereafter

3.2. Effects of enzymatic pretreatment on C, N, and P metabolism in fermentation

3.2.1. Carbon hydrolysis and metabolism

The metabolic profiles of elemental C in fermentation communities

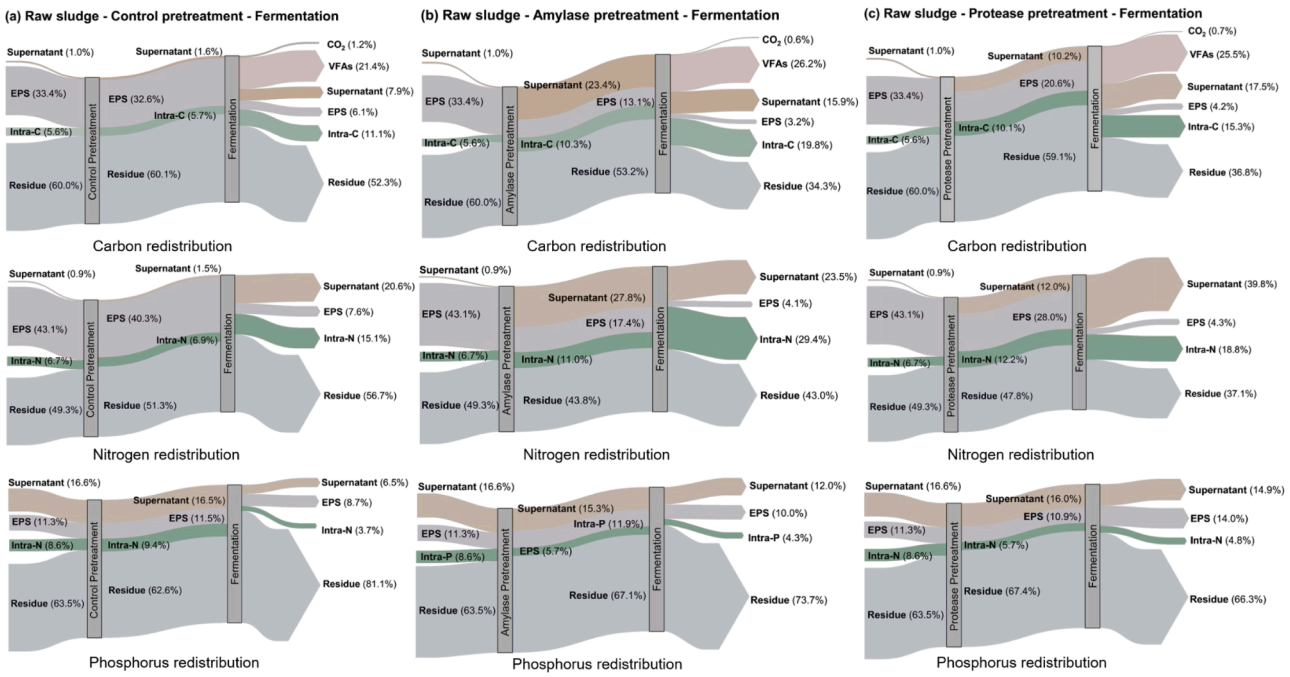

Fig. 1. Redistribution of carbon, nitrogen, and phosphorus among sludge components. (a) Control group; (b) Amy group; (c) Pro group. Intra-C/N/P represent the intracellular C, N or P.

were illustrated by transcriptome sequencing and gene-normalized transcript abundance analysis, and the high expression levels of organic carbon hydrolase genes were observed (Fig. 2a & b). In the fermentation, numerous glycohydrolase genes, i.e., α-amylase (EC 3.2.1.1), trehalose-phosphatase (EC 3.1.3.12), trehalase (EC 3.2.1.28), phosphoglucomutase (EC 5.4.2.6), endoglucanase (EC 3.2.1.4), and β-glycosidase (EC 3.2.1.4), were observed to upregulate in both Amy and Pro groups. The enzymes of trehalose and cellulose were reported to

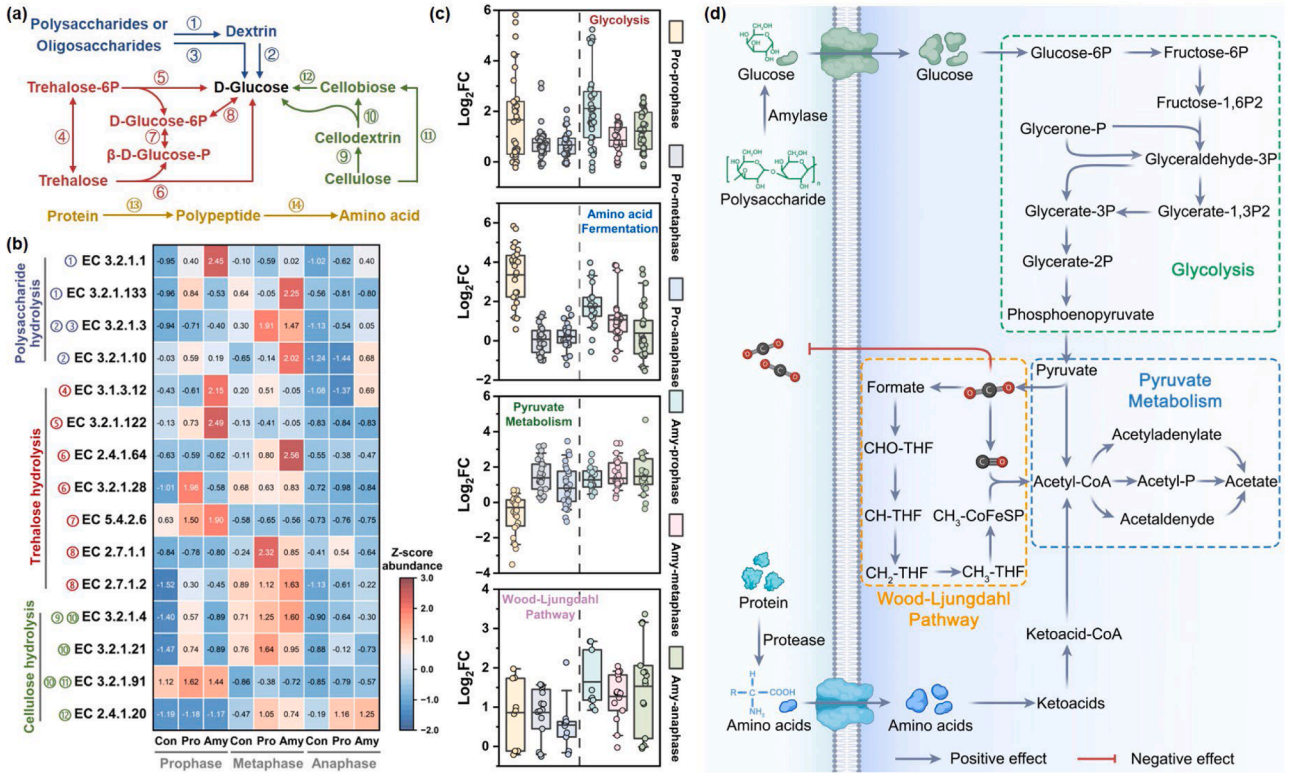

Fig. 2. Organic carbon transformations in enzymatic anaerobic fermentation systems. (a) Diagram of organic carbon decomposition. (b) Differential expression abundances of enzymes related to polysaccharide, trehalose, and cellulose hydrolysis in sludge fermentation. (c) Fold change (FC) in critical genes related to glycolysis, amino acid fermentation, pyruvate metabolism, and the Wood–Ljungdahl pathway. (d) Diagram of carbon metabolism involved pathways in enzymatic anaerobic fermentation systems.

play important roles in the decomposition of extracellular polysaccharides in sludge flocs (Yu et al., 2015). Meanwhile, several protease related genes were also observed to upregulate in the fermentation prophase (Figure S4). In the fermentation prophase, the amylase activity were determined to be 10.5 U/ml and 10.3 U/ml, and these were 1.67 and 1.64-fold higher than in the Con group. The protease activity were determined to be increased by 48.4 % in the Pro group and by 72.7 % in the Amy group (Figure S5). It was inferred that the generation of endogenous hydrolases involve in enzymatic fermentation, and the hydrolysis of organic carbon by them occurred therein.

Furthermore, hydrolyzed organic carbon, primarily sugars and amino acids, underwent further degradation and transformation within anaerobic microbes via key intra-C metabolic pathways, including glycolysis, amino acid fermentation, pyruvate metabolism, and the Wood–Ljungdahl pathway (Pan et al., 2024; Wang et al., 2016). It was observed that these four pathways showed different transcript responses towards enzymatic pretreatment (Fig. 2c). Upon introducing exogenous hydrolases, the genes linked to glycolysis were highly expressed in enzymatic fermentation systems, and 84.6 % and 91.7 % of genes exhibited higher transcript abundance in the Pro and Amy groups in fermentation prophase, respectively. Amino acids, as another micromolecular substrates for sludge fermentation, were also catabolized within the intracellular space. All genes in this pathway exhibited significantly greater transcriptional activity during prophase. Compared with the Amy group, the Pro group exhibited a stronger enhancement of amino acid fermentation during prophase. This effect might be attributed to the direct stimulation of this pathway by amino acids and peptides released by protease hydrolysis during pretreatment. The enhanced catabolism of glucose and amino acid efficiently generated pyruvate and ketoacid-CoA, and this effect triggered the transcriptional upregulation of pyruvate metabolism in enzymatic fermentation systems (Kierans and Taylor, 2024; Song et al., 2025). Among these, eight differential transcripts were highlighted and were observed to be upregulated in the Pro and Amy groups during metaphase and anaphase. Beyond organic carbon catabolism, the Wood–Ljungdahl pathway associated with CO_2 fixation was also observed to be more significantly active with enzymes introduction. Within this pathway, 40.0 % and 73.3 % of genes in the Pro and Amy groups exhibited significant upregulation ($Log_2FC>1$) as compared with that in the Con group, respectively. This included multiple key enzymes in CO_2 reduction pathways, such as formate dehydrogenase (FdhAB, CO_2→formate), formate tetrahydrofolate ligase (Fhs, formate→formyl-THF), and methylenetetrahydrofolate reductase (Met, CH_2-THF→CH_3-THF). (Fig. 2d). These findings demonstrate that exogenous hydrolases promotes intracellular carbon metabolism and transformation, particularly the amino acid fermentation pathway activated by protease, and this effect enhances intra-C fixation potential and reduces CO_2 release during anerobic fermentation (Fig. 2d).

3.2.2. Nitrogen transformation and metabolism

To investigate the relationship between enzymatic hydrolysis and N metabolism, the N transformation profiles within the fermentation communities were correlated, and the primary metabolic differences were predominantly observed in the fermentation prophase. For nitrogen reduction, genes encoding for nitrate reductase (NO_3^-→NO_2^-) were downregulated by over 66.1 % upon hydrolase introduction. In contrast, genes encoding for nitrite reductase (NO_2^-→NH_3) exhibited higher expression with the increases of 17.1 %–110.8 % (*nrfA*, EC 1.7.2.2) and 1973.3 %–2821.2 % (*nir*, EC 1.7.2.15) (Fig. 3a & b-1). Moreover, the aqueous NH_4^+-N concentrations increased significantly owing to the solubilization and disintegration of protein by enzymatic pretreatment (Figure S6). Thus, the ammonium transporter was regulated in response to the extracellular NH_4^+-N enrichment in the fermentation prophase. The transcript abundance of the ammonium transporter (amt family) was increased by 395.3 % and 581.9 % in the Pro and Amy groups, respectively (Fig. 3b-2).

The more significant nitrite reduction and ammonium transport effectively supported the synthesis of intra-N within the fermentation communities, and this effect further stimulated the glutamate synthesis pathway (Fig. 3a & b-3). Specifically, genes encoding for glutamine

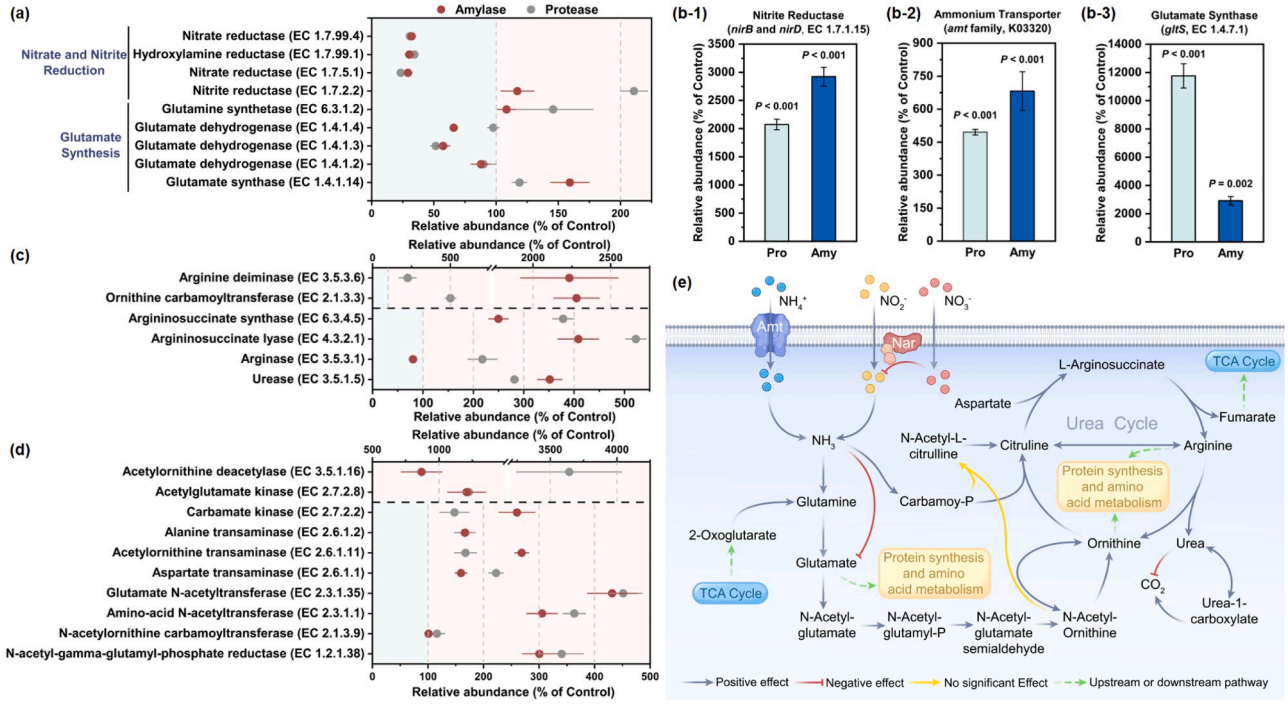

Fig. 3. Transcriptome analysis results of nitrogen metabolism involved functions. Relative expression levels of (a) critical enzyme genes for nitrate/nitrite reduction and glutamate synthesis; (b-1) ammonium transporter (K03320), (b-2) nitrite reductase (EC 1.7.1.15), and (b-3) glutamate synthase (EC 1.4.7.1); (c) critical enzyme genes for the urea cycle; (d) arginine biosynthesis in fermentation prophase. Control % = 100 %. (e) Diagram of nitrogen transformations involved pathways in enzymatic anaerobic fermentation systems.

synthetase (EC 6.3.1.2, NH_3→glutamine) were upregulated by 8.4 % in the Pro group and by 45.9 % in the Amy group. The genes encoding glutamate synthetases, particularly gltS (EC 1.4.7.1, glutamine→glutamate), also exhibited significantly higher transcriptional activity. Although the transcript abundance of glutamate dehydrogenase genes was slightly decreased by 3.2 %–34.1 %, the active expression of glutamine and glutamate synthetase genes suppressed this effect and remarkably enhanced glutamate synthesis. The stimulation of the glutamate synthesis module was reported to play important roles in driving the urea cycle and arginine biosynthesis pathway (Song et al., 2025; Xue et al., 2024). As expected, nearly all genes in urea cycle and arginine biosynthesis pathway showed increased expression in enzymatic fermentation (Fig. 3c & d). For example, key genes involved in the glutamate transformation (glutamate→N-acetyl-ornithine), including EC 2.3.1.1, EC 2.7.2.8, EC 1.2.1.38, and EC 2.6.1.11, were upregulated by 67.4 %–1123.9 % in the Pro and Amy groups. The gene coding argininosuccinate lyase (EC 4.3.2.1, L-argininosuccinate→arginine) showed the upregulation by 308.4 %–422.4 % accordingly. In living organisms, the urea cycle is central to ammonia homeostasis and ensures the adequate cellular N supply and efficient ammonia metabolism (Hu et al., 2023). Moreover, the urea cycle and arginine synthesis act as the gating pathways for polyamine and protein biosynthesis, and were reported to directly regulate the functional activity and cellular proliferation of organisms (Li et al., 2019; Oratz et al., 1983). The active NH_4^+-N conversion, as induced by enzymatic pretreatment, stimulates the synthesis of functional proteins and the enhancement of key functions in the subsequent fermentation community, such as the secretion of endogenous organic carbon hydrolases (Fig. 2b). These results indicate that enzymatic pretreatment improved the synthesis and bioconversion of organic N, especially amino acids, during the fermentation. This supports a functionally robust fermentation community through improved protein availability and synthesis (Fig. 3e). Among the pretreatments, protease exhibited a more significant promoting effect on N metabolism, primarily owing to stimulation by N-hydrolyzing products. It was reported that the release of appropriate amounts of ammonia improved anaerobic fermentation performance (Lauterböck et al., 2012; Procházka et al., 2012), except at extremely high ammonia concentrations of >6000 mg NH_4^+-N/L with inhibitory effects. The relativity between NH_4^+-N release and the N transformation within the fermentation communities, as documented by metatranscriptomics in this study, has not been reported before and potentially advance us the understanding and optimization of sludge fermentation process.

3.2.3. *Phosphorus turnover and metabolism*

To explore the P recovery efficiency and feasibility by enzymatic anaerobic fermentation, further analysis focused exclusively on genes encoding proteins involved in the microbial P turnover, including the regulation, solubilization, and uptake of extracellular P sources. It was observed that the Pro and Amy groups exhibited the strong similarity in the fermentation prophase. The majority (~80 %) of these genes exhibited significantly higher transcript abundance ($Log_2FC>1$) as compared to that in the Con group (Fig. 4a). This suggests that enzymatic hydrolysis considerably boosted the secretion of microbe-derived enzymes, including alkaline phosphatase (encoded by *phoD*), acid phosphatase (*phoN*), phosphonatase (*phnX*), and phosphodiesterase (*phnP*). These beneficial effects improved the release of free orthophosphate from recalcitrant organic P in sludge (Grafe et al., 2018; Rossolini et al., 1998). Meanwhile, under the regulation of the two-component system, i.e., *phoB, phoR*, and *phoU*, the expression levels of genes encoding the inorganic P transporter (Pit) and the multimeric ABC-type P specific transporter (PstABCs) were also observed to increase significantly, and this potentially enhanced bacterial P uptake and mitigated intracellular P deficiency (Wanner, 1993). Interestingly, in the Amy group the genes associated with inorganic P solubilization were upregulated significantly during fermentation anaphase. This might be attributed to the formation and accumulation of organic acids such as

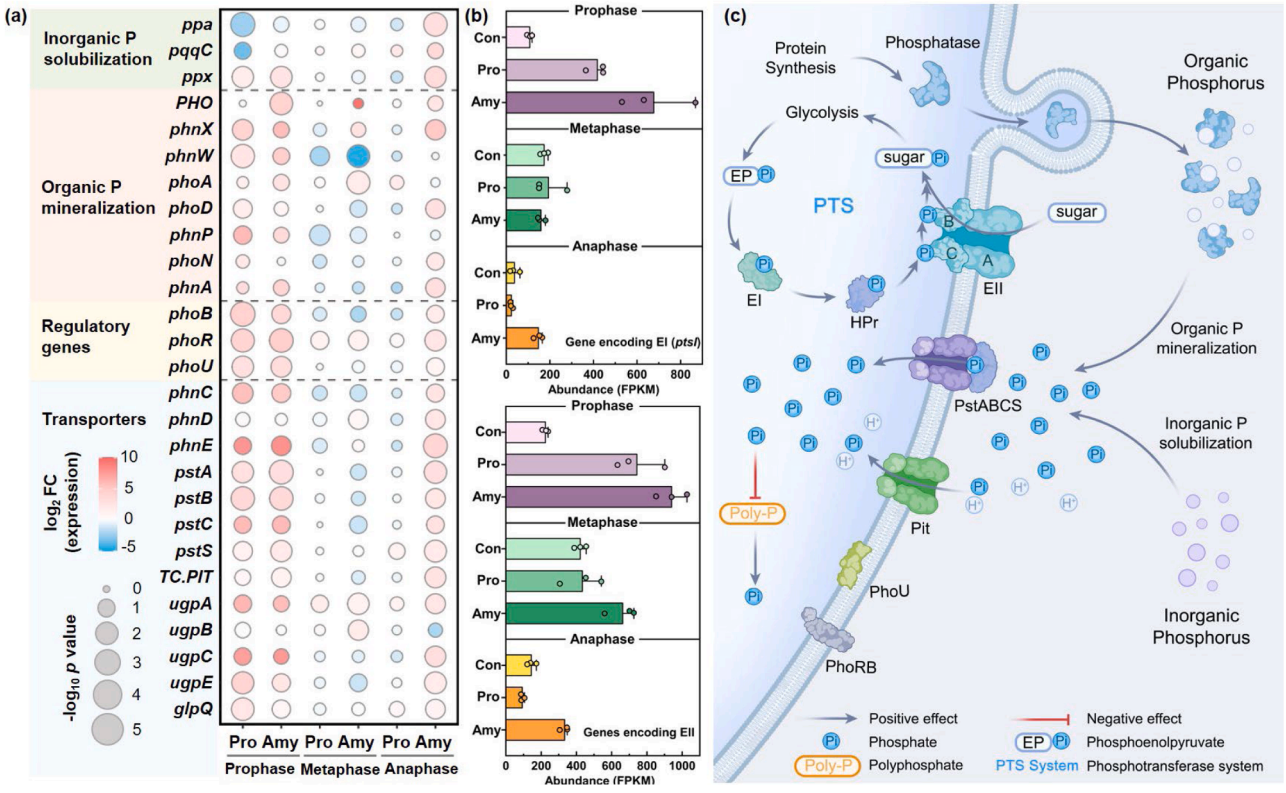

Fig. 4. Transcriptome response of phosphorus metabolism involved functions. Relative expression levels of critical genes for (a) inorganic phosphorus solubilization, organic phosphate mineralization, and phosphate regulation and transport, and (b) phosphotransferase system (PTS). (c) Diagram of phosphorus metabolism involved pathways in enzymatic anaerobic fermentation systems.

gluconic acid, and this effect stimulated the microbial solubilization of recalcitrant inorganic P (Liang et al., 2020). The microbial P uptake and cycling processes was also enhanced in the subsequent fermentation anaphase accordingly. Regarding to P polymerization, enzymatic hydrolysis enhanced the degradation rather than the synthesis of polyphosphate in anaerobic fermentation (Figure S7). These results highlighted the involvement of microbial-assisted P transformation in enzymatic fermentation of sludge, and offered insights into the bioaccessibility of various P forms and P recovery accordingly.

In addition to microbe-driven P uptake and recycling, intracellular phosphoryl group transfer plays a prominent role in substrate metabolism and energy generation (Kornberg et al., 2000; Xu et al., 2023). As a representative phosphorylation cascade, the genetic potential of phosphoenolpyruvate–carbohydrate phosphotransferase system (PTS) was analyzed to investigate the effects of enzymatic hydrolysis on the intracellular phosphoryl group transfer and microbial metabolism. As core PTS components, the Enzyme I (EI, encoded by *ptsI*) and Enzyme II complex (EII, comprising EIIA, EIIB, and EIIC/D) exhibited significantly increased transcriptional activities upon enzymatic pretreatment (Fig. 4b). Specifically, the transcript abundance of ptsI increased by 288.2 % and 535.1 % in the Pro and Amy groups. The corresponsive genes encoding EII increased by 233.0 % and 318.4 % during fermentation prophase. The important roles of phosphate in cellular components and metabolic processes have been widely reported (Reizer et al., 1996; Walton et al., 2023; Westheimer, 1987). The PTS involves in the uptake of diverse carbohydrates and the regulation of carbon catabolite repression, and this effect has been extensively illustrated in different model microorganisms (Görke and Stülke, 2008; Long et al., 2017; Rojo, 2010). These results indicated the positive effects of enzymatic hydrolysis on carbohydrate uptake and metabolic versatility optimization as mediated by intracellular phosphoryl group transfer, and this activated intracellular P cycle may affect the C biological cycle accordingly (Fig. 4c). Overall, the introduction of protease and amylase promoted P solubilization and release from sludge residue, as well as microbial-mediated P turnover. Upon enzymatic pretreatment, P metabolism might be more strongly coupled with polysaccharide uptake, especially in the Amy group, thereby exhibiting close cooperation with C metabolism. It was noted that upon the introduction of enzymes, nearly all genes relevant to sulfate transport and assimilatory sulfate reduction exhibited increased expression levels (Figure S8, Figure S9 & Supplementary Results and Discussion).

3.3. Recovery of C, N, and P by enzymatic anaerobic fermentation

To further illustrate the effects of enzymatic pretreatment on the resources recovery by anaerobic fermentation, the production of struvite, i.e., as precipitates to harvest ammonia and phosphate, and SCOD as carbon sources for denitrification were quantitatively compared. Upon anaerobic fermentation, the total phosphate concentrations in supernatants increased from 45.4 mg/L for the Con group to 62.0 mg/L for Pro group and 60.2 mg/L for Amy group, and the recovery of N and P as struvite was increased by 13.7 % and 19.0 % to 21.1 mg/g TS and 22.1 mg/g TS accordingly (Fig. 5a). It was noted that enzymatic pretreatment rarely enhanced P release (Fig. 1), and the elevated phosphate concentrations were attributed to the positive effects towards P metabolism and turnover involved in fermentation (Grafe et al., 2018). The more significant release of phosphate and ammonia improved their recovery as struvite upon enzymatic anaerobic fermentation.

After struvite crystallization, the SCOD in the supernatant were respectively determined to be 15,513 mg/L and 17,447 mg/L in the Pro and Amy groups, i.e., 1.3- and 1.4-fold higher than that in Con group (Fig. 5b). The growth curves of *P. denitrificans* in the Pro and Amy groups, as indicated by kinetic OD_{600} increase, were observed to be significantly higher than that in the Con group and to be slightly lower than the commercial acetate (Fig. 5c). After 30-hrs cultivation, the observed maximum specific growth rates of *P. denitrificans* in the Pro and Amy groups were higher to be 0.12 h^{-1} and 0.13 h^{-1} than that in the acetate group (0.09 h^{-1}), whereas that in the Con group was 0.18 h^{-1}. At initial NO_3^--N concentration of as high as 750 mg/L, the residual NO_3^--N concentrations were below 35.0 mg/L with the enzymatic-pretreated supernatants as carbon sources, whereas that in the Con group was observed to be 187.3 mg/L accordingly (Fig. 5d). The good assimilability may be primarily ascribed to the combined effects of enzymatic pretreatment and fermentation towards sludge EPS solubilization, the metabolism and transformation of slowly biodegradable organic matter (Cao et al., 2019; Kang et al., 2018). To further explore the utilization

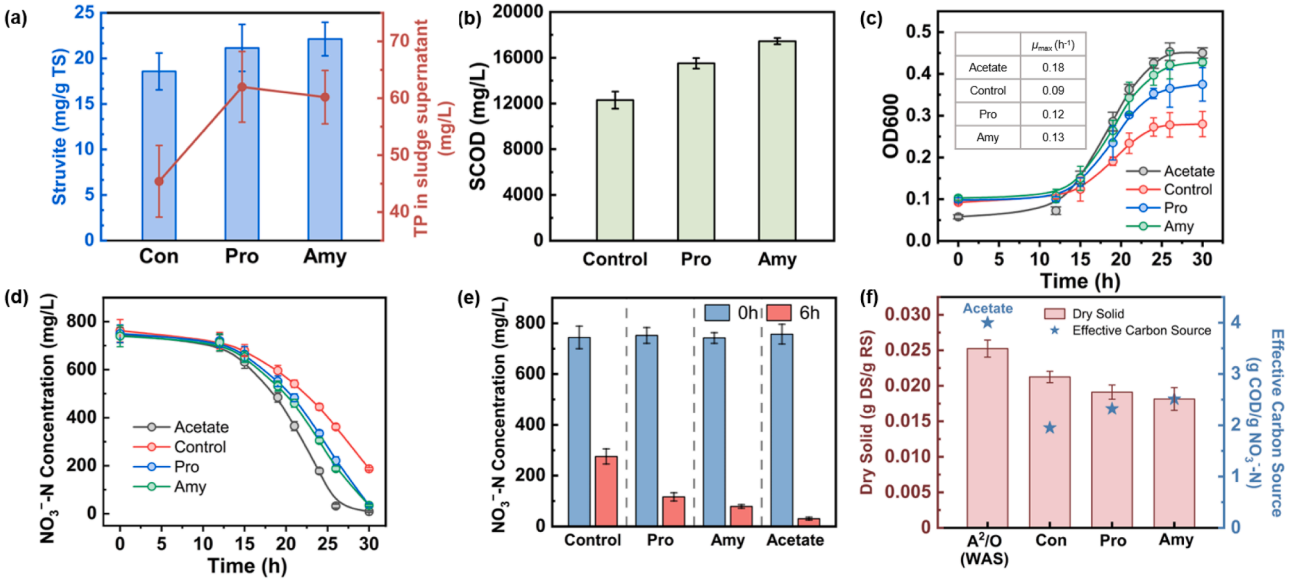

Fig. 5. Resource recovery of carbon, nitrogen, and phosphorus in enzymatic anaerobic fermentation systems. (a) Total phosphorus concentration in the sludge supernatant after fermentation and the efficiency of struvite recovery; (b) SCOD concentration in fermented sludge; (c) OD_{600} of *P. denitrificans* cultured with sludge-derived carbon source during denitrification, with acetate as the control; (d) Temporal variation of NO_3^--N concentration in denitrification systems of *P. denitrificans* with different carbon source; (e) NO_3^--N concentration variations in denitrification sludge systems with different carbon sources; (f) Dry solids of WAS and sludge upon fermentation and carbon source return, and the effective carbon source of supernatant for denitrification system.

feasibility in realistic WWTPs, the denitrification sludge was used to compare the denitrification performance among these different carbon sources (Fig. 5e). Upon 6-hrs denitrification, NO_3^--N removal was observed to be 93.9 % in the Pro group and 95.3 % in the Amy group, whereas that in Con group was lower to be 75.1 %. Additionally, the effective carbon source was determined to be 2.32 g COD/g NO_3^--N in the Pro group and 2.51 g COD/g NO_3^--N in the Amy group, and was near to the required C/N ratio of 2.86 : 1 to achieve theoretically complete denitrification (Fig. 5f). It was noted that in the Con group the C/N ratio was much lower to be 1.95 g COD/g NO_3^--N. These results indicated that enzymatic anaerobic fermentation enhanced the utilization efficiency and economic benefit with regard to waste sludge reclamation as carbon sources.

In addition to the substitution of commercial acetate to reduce costs, enzymatic anaerobic fermentation also reduced the dry sludge solids due to the recycling of supernatants with extremely high SCOD concentrations. On the basis of theoretical mass balance calculation (Table S3), the dry sludge solids were determined to be reduced by 24.3 %–28.1 % as compared to WAS from the conventional A^2/O process (Fig. 5f). To further assess the environmental impacts, LCA was used to compare the environmental benefits between the conventional anaerobic fermentation and enzymatic anaerobic fermentation (Figure S10). Results indicated that the Pro and Amy groups exhibited significantly lower GWP at −194.6 Kg GWP/t DS and −311.0 Kg GWP/t DS, respectively, whereas the GWP value in Con group was calculated to be 3.6 Kg GWP/t DS. These benefits may be attributed to the reduced sludge volumes for landfill and the lower non-point greenhouse gas emissions involved in enzymatic anaerobic fermentation. Additionally, the key pollution and toxicity indicators, i.e., AP, EP, FAETP, TETP, HTP, POCP, ODP, and MAETP, of enzymatic anaerobic fermentation also indicated the minimal environmental negative impacts. Furthermore, the economic cost analysis indicated that the introduction of amylase significantly increased the economic benefit of sludge treatment by 57.4 % (Figure S11). In contrast, protease reduced the net economic benefit and rendered the enzymatic anaerobic fermentation nearly cost-neutral, and this might be attributed to the higher energy demand associated with protease pretreatment. Given that protease hydrolysis promotes the release of nitrogen-rich fermentation products, the targeted recovery of high-value sludge-derived proteins or amino acids may yield greater economic benefits (Xiao and Zhou, 2020). In future engineering applications, producing enzymes from waste biomass might represent a cost-effective strategy to further improve the economic feasibility of sludge valorization (Gupta et al., 2016). These results highlighted the potential environmental and economic benefits of enzymatic anaerobic fermentation for sludge treatment, resources harvesting, and final disposal. Further piloting experiments are necessary to comprehensively evaluate its technical feasibility and economic viability for large-scale engineering applications.

4. Conclusion

This study focuses on the recovery of valuable C, N, and P resources from WAS by enzymatic anaerobic fermentation, and the redistribution and cycling of these elements and the microbial metabolic behaviors involved in are carefully investigated. Results indicate that enzymatic hydrolysis significantly increases the proportions of C and N in sludge supernatants by 21.8 %–26.3 %, and the subsequent fermentation increases these proportions of C, N, and P in the outer components by 7.4 %–18.0 %. Interestingly, the introduction of amylase and protease significantly enhances the metabolism of C, N, and P such as organic carbon catabolism, NH_4^+-N conversion, and P solubilization, in the subsequent anaerobic fermentation. The positive effects of enzyme improve the recovery efficiency of the C, N and P as dentification carbon source and struvite precipitates. In addition to these benefits, enzymatic anaerobic fermentation decreases the sludge production by 24.3 %–28.1 % as dry sludge solids as compared to conventional A^2/O process, and the GWP was calculated to be decreased from 3.6 Kg GWP/t DS in Con group to −194.6 and −311.0 Kg GWP/t DS in the Pro and Amy groups, respectively. This study advances the understanding of the C, N and P recovery potential and elemental metabolism within the fermentation microbiome, and provides practical insights to harvest multiple valuable resources from sludge and organic solid wastes towards the low-cost, value-added, and CO_2 emission reduction biorefinery.

CRediT authorship contribution statement

Ge Song: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Shunan Zhao:** Resources, Methodology, Investigation, Formal analysis. **Kai Zhao:** Methodology, Investigation. **Ruiping Liu:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Chengzhi Hu:** Writing – review & editing. **Mark C.M. van Loosdrecht:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.resconrec.2025.108578.

Data availability

Data will be made available on request.

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