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# Bioaugmentation of microalgae fermentation with yeast for enhancing microbial chain elongation: *In-situ* ethanol production and metabolic potential

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ABSTRACT

Anaerobic microalgae fermentation, leveraging its cost-effectiveness and the adaptability of mixed cultures, holds promise for carboxylate biosynthesis. Microalgae, with their abundant carbohydrates and proteins, stand out as an optimal substrate for this process among various options. Furthermore, microalgae fermentation not only shows the potential to mitigate risks associated with algae blooms but also aligns with the need for sustainable practices. However, the limited utilization rate of microalgae in anaerobic fermentation poses challenges to achieving high production rates of desired products. In this study, we implemented a bioaugmentation process with yeast to enhance carboxylate production performance of microalgae fermentation. The results demonstrated a fourfold increase in carboxylate yield with the addition of yeast. *In-situ* ethanol production facilitated the conversion of short-chain carboxylic acids into medium-chain carboxylates, achieving a yield of 46.3 mM-C/g VS. The presence of yeast significantly enhanced substrate utilization from 20 % to 80 %, steering the metabolic pathway towards chain elongation. Metagenomic analysis further revealed metabolic shifts following yeast addition, particularly an increased abundance of genes involved in acetyl-CoA production. Notably, the aldehyde:ferredoxin oxidoreductase (AOR) pathway emerged as a key driver in butanol production. These findings highlight the improved performance of anaerobic microalgae fermentation with yeast, enabling efficient production of higher value bioproducts while eliminating the need for external electron donors.

## 1. Introduction

The microalgae-based biofuel production platform has garnered widespread recognition as a highly promising and self-sustainable technology [1,2]. This recognition stems from its unique ability to directly harness solar energy through photosynthesis for the efficient conversion of carbon dioxide [3]. Microalgae exhibits significantly higher productivity levels compared to other biological technologies, further enhancing its appeal [4]. This high production potential is attributed to the ability of microalgae to harness photosynthesis for carbon dioxide fixation and absorb nutrients from the aquatic environment. In this process, microalgae produce abundant lipids as a form of stored energy to support their growth [5]. Consequently, microalgae have emerged as versatile cell factories with applications spanning feed, agriculture, and energy sources [6].

The biorefining of microalgae within a circular loop, with the aim of maximizing resource recovery, plays a crucial role in the production of biobased products, encompassing the cultivation, harvesting, and downstream processes of microalgae [7]. This approach holds both economic and environmental viability. However, the long-term sustainability of this carbon–neutral system may face challenges due to the substantial energy and nutrient resources required for the conversion of biomass to biofuel [8]. The supply of carbon dioxide and the extraction of lipids often require a significant amount of additional energy, while the costs associated with these processes are frequently not offset by the sales price of the final product [9]. The complexity of operating

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management in the microalgae biorefining process is further compounded by the requirement for pure cultures [10]. Moreover, the proper treatment of waste microalgae after lipid extraction is a critical consideration [6]. In this case, utilizing waste microalgae as a substrate for anaerobic fermentation to produce valuable chemicals represents a promising strategy to bolster the sustainability of microalgae biorefining, effectively closing the nutrient recovery loop [11].

Carboxylate platforms represent inherent microbial processes that convert biomass into carboxylates, obviating the requirement for intricate dewatering procedures or additional chemical extraction [12]. For example, Chang et al. integrated the photosynthesis of microalgae with anaerobic fermentation to achieve energy recovery with 10.80 kJ/L [13]. This coupled process exhibited a great potential for biorefining of microalgal biomass due to the high organic contents in microalgae due to the photosynthesis of microalgae [14]. Microalgal species are generally characterized by 20–30 % carbohydrates, which lack lignin, facilitating their conversion to monosaccharides compared to lignocellulosic materials [15–19]. Furthermore, 40–52 % high-quality protein, with 35–50 % being digestible makes them suitable for the substrate for carboxylate platforms [18]. Given the vast number of microbial species in open-culture fermentation, anaerobic microalgae fermentation can efficiently utilize this complex biomass substrate and estimate the need for sterilization.

Microbial chain elongation (CE) is an essential process in carboxylate platforms aimed at improving the value of products, where specific microorganisms utilize carboxylates such as acetate, in conjunction with an electron donor (e.g., ethanol) to produce longer-chain carboxylates, including caproate, heptanoate, and caprylate [19,20]. In the 1930 s, a novel species called *Clostridium kluyveri* was isolated and has since become the model microorganism for the CE process [21,22]. The CE process in *C. kluyveri* is accomplished through reverse $\beta$-oxidation (rBOX), leading to a two-carbon elongation per cycle [23,24]. Although rBOX is thermodynamically favorable, it necessitates the presence of energy-rich and reduced compounds as electron donors, as well as a hydrogen partial pressure of at least $10^{-1}$ kPa to prevent forward $\beta$-oxidation [25,26]. Other electron donors, such as lactic acid or carbon monoxide, have been discovered in microbial CE processes [27,28]. However, using additional chemicals to produce medium-chain carboxylates is hindered by their high cost and resource consumption.

Leveraging the robust ethanol production capacity of yeast, this study integrates yeast with anaerobic microalgae fermentation to produce ethanol *in situ*, serving as the electron donor for the synthesis of medium-chain carboxylic acids (MCCAs). The sustainability of this approach is enhanced by the yeast's ability to produce ethanol within the reactor, thereby reducing the need for external ethanol inputs and optimizing resource utilization. This investigation explores the feasibility of this innovative system for efficient MCCA production. Furthermore, metagenomic analysis was utilized to unravel the microbial community structure and metabolic potential, offering deeper insights into the dynamics and interactions driving this novel fermentation process. This integrated approach not only highlights the potential of yeast in biotechnological applications but also advances our understanding of microbial interactions in bioaugmented systems.

## 2. Materials and methods

### 2.1. Source of microalgae, inoculum and yeast

Microalgae were obtained from Huachu Trading Co., Ltd. (Henan, China) and were stored in a refrigerator at −20 °C before use. The main species of microalgae was *Chlorella*. The total suspended solids (TSS) of microalgae were 52.43 ± 0.06 g/L and its volatile suspended solids (VSS) were 48.82 ± 0.04 g/L. Prior to the tests, the microalgae were diluted to a VSS concentration of 15 g/L using tap water. The total chemical oxygen demand (COD) of diluted microalgae was 22.56 ± 10.64 g/L, which had a composition of 55.1 % ± 5.2 % protein, 17.5 % ± 3.1 % total carbohydrates.

The inoculum used in the study was anaerobic digestion sludge (ADS) and obtained from our lab-scaled mesophilic reactor, which was used to treat excess activated sludge. To remove residual organics, the sludge was self-digested for a minimum of a month without any external organic supply. The final inoculum had TSS and VSS concentrations of 46.26 ± 0.29 g/L and 20.01 ± 0.16 g/L, respectively. The commercial dry baker's yeast used in the study was purchased from Angel Company (China) and consisted primarily of *Saccharomyces cerevisiae*.

### 2.2. Anaerobic microalgae fermentation protocol

Anaerobic microalgae fermentation was conducted using a short-term batch experiment in 150 mL serum flasks. Specifically, two yeast groups were prepared by combining 36 mL of microalgae, 64 mL of ADS, and 2 g dry yeast, resulting in a total volume of 100 mL. This achieved a substrate-to-inoculum volatile solids (VS) ratio of 0.5. Two control groups were also included, identical to the experimental groups but without yeast. To assess the fermentation performance of the inoculum and *saccharomyces cerevisiae*, four blank groups were set up, replacing microalgae with tap water. The NaOH and HCl solutions (3 M) was used to adjust pH of fermentation broth to 5.0 ± 0.1. After each sampling, the pH was also adjusted manually to maintain 5.0. The pH was relatively stable throughout the fermentation (<0.5 units change), indicating limited fluctuations during the experiment. The pH of 5.0 was chosen based on its demonstrated optimal chain elongation performance in the pre-experiment. To maintain anaerobic conditions, pure nitrogen was purged to the headspace of reactor for 15 min after sampling. Subsequently, all bottles were placed in an incubator shaker at 35.0 ± 0.1 °C and 130 rpm for the fermentation process.

### 2.3. Chemical analytical methods

The head gas sample (1 mL) was collected by syringe with seal valve and then was injected into a gas chromatograph (GC-112A, China) equipped with a thermal conductivity detector (TCD) to analyses the concentration of $H_2$, $CO_2$ and $CH_4$. At the same time, a 2 mL sample of the liquid was collected, then centrifuged and filtered. To ensure that the carboxylic acids remained as free molecules, 5 μL/mL of formic acid (HCOOH) was added to the sample. A gas chromatograph (GC-2010 Plus, SHIMMADZU, Japan) was used to determine the carboxylic acids and butanol. This gas chromatograph was equipped with a flame ionization detector (FID) and a capillary column (SH-Stabllwax-DA, 30 m × 0.32 mm × 0.25 μm).

The chemical oxygen demand (COD) was measured using the fast digestion-spectrophotometric method [29]. The VS and TS were analyzed using the same methods as previous research [30]. The concentration of protein and reducing sugar in the microalgae was determined by reformative Lowry method and the 3,5-dinitrosalicylic acid colorimetry [31,32].

### 2.4. DNA extraction and metagenomic sequencing

Twenty milliliters anaerobic sludge samples collected from two duplicate fermentation bottles of both the control and experiment groups were evenly mixed for genomic DNA extraction. Following the completion of experiment, the liquid sample underwent harvesting through centrifugation. The E.Z.N.A. Soil DNA Extraction Kit Genomic DNA extraction kit was used to extract genomic DNA in accordance with the manufacturer's guidelines. To assess the purity of the extracted DNA, absorbance measurements ($\lambda = 260$ nm, $\lambda = 280$ nm) were conducted using a NanoDrop spectrophotometer (USA). Subsequently, the Covaris M220 (Covaris, USA) was utilized to fragment the DNA to chieve a target size of approximately 400 bp. Subsequently, the NEXTFLEX™ Rapid DNA-Seq Kit was used to metagenomic library preparation. The

resulting DNA library was subjected to paired-end sequencing (2 × 150 bp) on the Illumina Hiseq4000 platform (Illumina, USA).

### 2.5. Metagenomic analysis

Metagenomic sequencing reads underwent meticulous processing. Fastp (v0.20.0) was employed for trimming and adaptor removal [33]. To ensure the accuracy of metagenomic analysis by preventing interference from the yeast genome, Bowtie 2 (v.2.3.5.1) was used to remove the genome of *Saccharomyces cerevisiae*, obtained from NCBI (GCF_000146045.2) [34]. Subsequently, the profiling of the microbial community composition was analysed by MetaPhlAn 4.0 (v4.0.6) [35]. For an in-depth exploration of metabolic potentials, the trimmed reads were assembled with MEGAHIT (v1.1.2) [36]. Prodigal (v2.6.3) was employed for predicting protein-coding genes [37]. All predicted genes were merged and clustered with CD-HIT (v4.6.1, −aS 0.9, −c 0.9) to construct non-redundant gene sets [38]. Reads after quality control were mapped back to the non-redundant gene sets with 95 % identity using salmon (1.10.2) [39], and the reads count were used to calculate the gene abundance, expressed as transcripts per kilobase per million mapped reads (TPM) in each sample. All of the genes were taxonomically aligned to EggNOG and dbCAN2 by Diamond v0.8.35 [40–44]. The clean data was uploaded to NCBI under the BioProject No. PRJNA1144057.

## 3. Results

### 3.1. Yeast bioaugmentation effectively prolonged the carbon chain of fermentation products into medium-chain carboxylic acids

The final concentrations of the main products in the control and yeast groups are shown in Fig. 1A and 1B. Microalgae showed a feasible fermentation performance for carboxylate production. In the single microalgae fermentation, the total concentration of carboxylate was 54.0 ± 9.9 mM-C during 25 day-fermentation. The addition of yeast significantly increased the concentration to 184.6 ± 16.2 mM-C. Among them, acetate, butyrate, and valerate concentrations in the yeast group were 3.9-fold, 2.1-fold, and 2.4-fold higher, respectively, compared to the control group. This enhancement would be attributed to the *in-situ* production of ethanol by the yeast fermentation. Fig. 1C illustrates the ethanol concentration in the yeast group during the first nine days, peaking at 28.2 ± 0.4 mM-C. The concentration of reducing sugar in microalgae was 3.95 g/L. In the yeast group, the ethanol yield was 0.45 g/ (g reducing sugar) near the theoretical ethanol yield of *saccharomyces cerevisiae* which indicated the yeast plays a crucial role in the *in-situ* production of ethanol.

The consumption of ethanol during subsequent fermentation was linked to the accumulation of butyrate and caproate, signifying the involvement of the rBOX in the chain elongation of carboxylic acids. In the yeast group, acetate accumulation was noted, with concentrations reaching 93.93 ± 14.80 mM-C, which represented approximately 37 % of the COD fraction in the final products. This accumulation of acetate can be attributed to the inhibition of the chain elongation process under

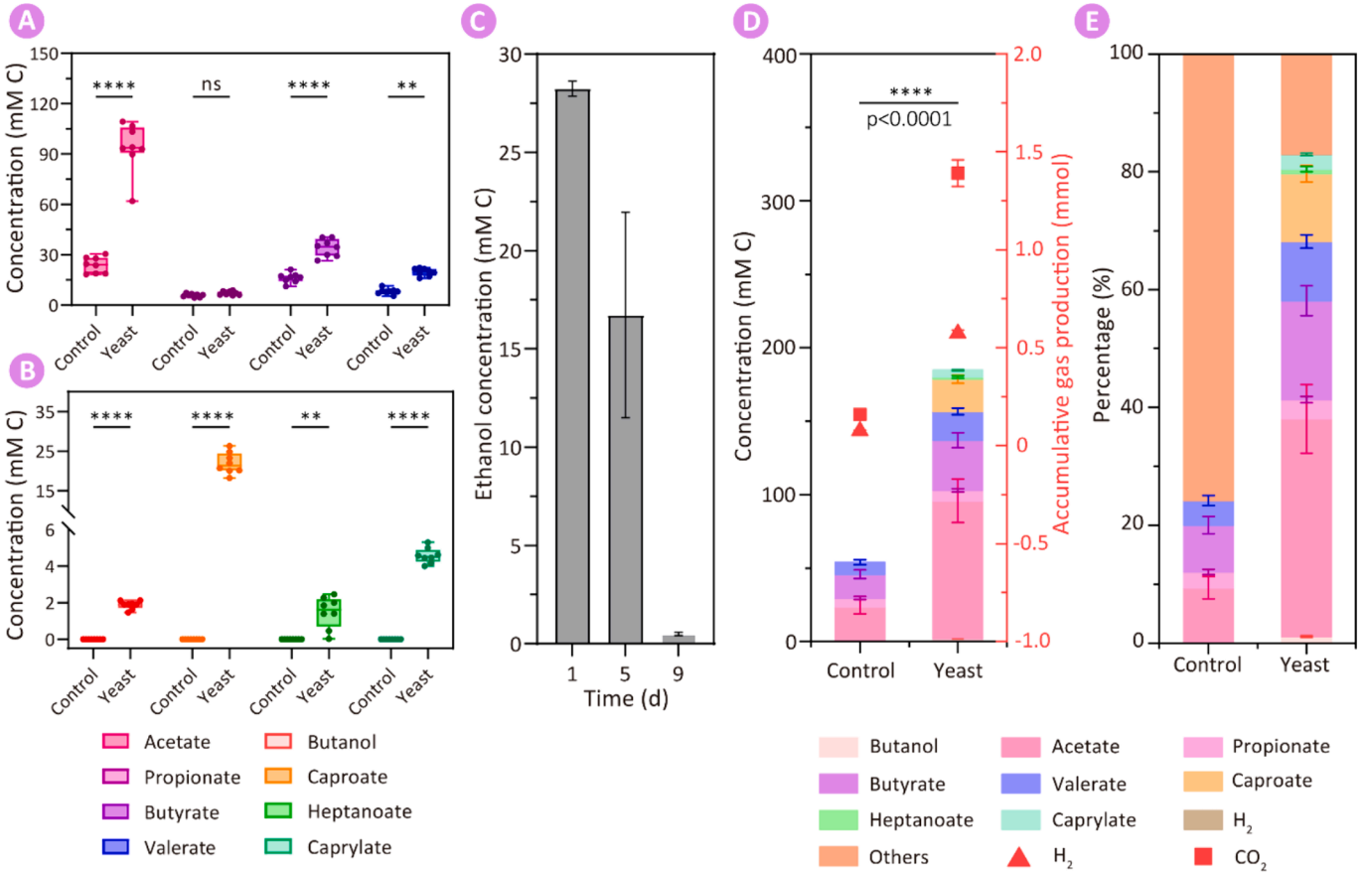


**Fig. 1.** The comparison of (A) SCCA production and (B) MCCA/alcohol production between control and yeast group. (C) The ethanol concentration of the yeast group within the initial nine days. (D) The product spectrum of microalgae fermentation with and without yeast. (E) The product distribution normalized with TCOD input of substrate. The statistical difference between control and yeast group is indicated as asterisk mark above the plots. (ns: P>0.05, *: 0.05 ≥ P>0.01, **: 0.01 ≥ P>0.001, ***: 0.001 ≥ P>0.0001, ****: p ≤ 0.001).

acidic conditions (pH 5.0) and the toxicity of the acids [43]. A transient acetate accumulation was observed in our research, possibly from ethanol oxidation [44]. The produced ethanol serving as the electron donor to elongate the carboxylic acids into the longer carbon chain carboxylic acids, particularly caproate (from 0 to 21.9 ± 2.7 mM-C) and caprylate (from 0 to 4.6 ± 0.4 mM-C). Besides, the butanol production was also observed in the yeast group, with 1.9 ± 0.2 mM-C. The butanol production would also be attributed to the ethanol consumption. In this process, ethanol can serve as the reducing power to transform butyrate into butanol via butanol dehydrogenase (*bdh*) or alcohol dehydrogenase (*adh*) under reduced environments [45,46].

In the control groups (n = 2) without added yeast, the fermentation broth exhibited the presence of SCCA metabolites, while no medium-chain carboxylic acid (MCCA) was detected throughout the 25-day fermentation period. However, a concentration of 0.35 mM-C butyrate was still detected, which is lower than the 0.75 mM-C butyrate observed in the control group. These results suggest that while some chain elongation activity persists in the control group, it is lower compared to the yeast group. The lack of sufficient electron donors is responsible for the absence of MCCA production in the control group. The absence of significant metabolites in the absence of added yeast, along with the carbon and electron balances, supports MCCA and butanol produced through CE facilitated by added yeast, instead of the oxidation process from the microalgae biomass.

The addition of yeast significantly increased the fermentation performance, the total concentration of carboxylic acids increased by 4-fold compared to control group (Fig. 1D). In addition, the accumulative hydrogen production was also increased from 0.08 ± 0.01 mmol to 0.58 ± 0.02 mmol, indicating the better chain elongation performance in the yeast group. Notably, the enhanced carbon dioxide production was also detected in the yeast group, indicating the ethanol production was mainly due to the yeast fermentation. Whatever, the production of butyrate and valerate were still produced via chain elongation, however the electron donor was some amino acid hydrolyzed from proteins in the microalgae and the lower reduced power hindered the carbon chain further elongating [47].

Generally, yeast exhibit higher ethanol production rate compared to bacteria, which implies that yeast can convert a greater proportion of substrates into products. Consequently, incorporating yeast can enhance the substrate utilization rate, thereby also increasing the carboxylate concentration. To verify this hypothesis, the COD fraction was then calculated. Fig. 1E demonstrated the COD fraction of the main fermentation products. During the period of the batch experiments, the control groups converted 24.21 % ± 0.55 % of total substrate COD to carboxylic acids. In comparison, yeast group utilized 83.03 ± 0.80 % of microalgae COD to ferment. Specifically, the average conversion of COD in microalgae to MCCAs was 14.79 ± 0.22 %, and MCCAs accounted for 18.43 ± 1.83 % of the total carboxylic acids produced. The results indicated that the addition of yeast increased the substrate utilization rate and direct the metabolic potential towards chain elongation.

### 3.2. Yeast bioaugmentation shaped microbial community structure for chain elongation

The community composition at the phylum level in the control and yeast groups is presented in Fig. 2. Specifically, the microbial community structures were primarily influenced by the proliferation of bacteria belonging to the *Firmicutes* phylum, attributed to ethanol production, resulting in a rise in relative abundance from 3.0 % to 17.2 %. *Firmicutes* has been previously documented to exhibit high prevalence in chain elongation systems fueled by ethanol and carboxylic acids [19]. The enrichment of *Firmicutes* has consistently been observed in lab-scale bioreactors engaged in chain elongation, utilizing both simple substrates and complex feedstocks. In addition to *Firmicutes*, other phyla displayed varying degrees of increase, except for *Bacteroidetes* (decreased from 62.4 % to 26.5 %) and *Ignavibacteriae* (decreased from

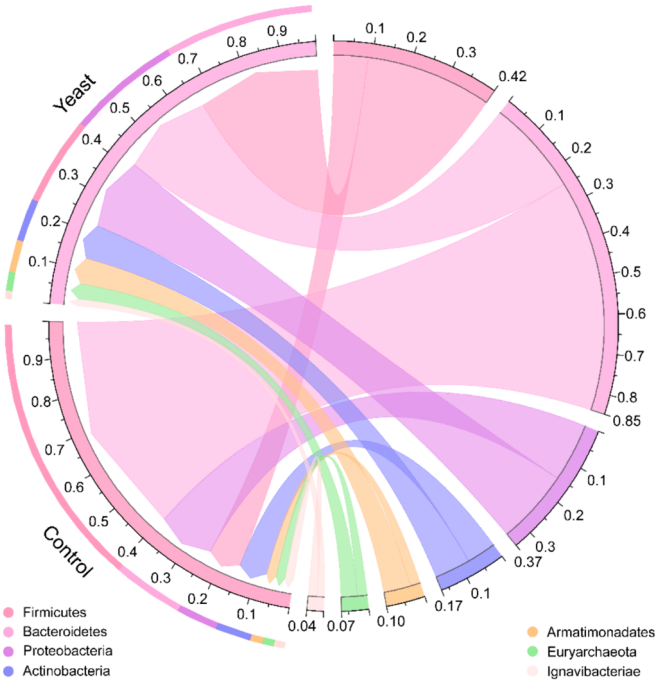


**Fig. 2.** The microbial structure of control and yeast group at the end of fermentation under Phylum level. The abundance was obtained by using MetaPhlAn 4 from trimmed reads as input data.

2.3 % to 1.8 %).

Given the community composition with the addition of yeast, the differences of microbial structure between control and yeast groups at Genus level was further examined (Fig. 3A). The most of genus have been enriched at different extent especially the acidogenic microbes including *Clostridium, Lactococcus*, *Enterococcus* etc. Notably, *Clostridium* belongs to *Firmicutes* have significant increased from 1.7 % to 8.8 % due to its chain elongation ability [23]. After investigating the specific species, one identified species (Fig. 3B), *C. butyricum* was significantly increased in relative abundance under yeast-existence CE. *C. butyricum*, is a strictly butyrate-producing bacterium [48]. The increased acidogenic microbes explained the enhanced performance for carboxylic acid production. In addition, some reported gas-independent Genus have also enriched in the yeast groups such as *Thermomonas* which can utilize hydrogen to stabilize pH level of fermentation borth [49]. *Methanothrix* was also increase from 1.0 % to 1.7 % which was reported to use carbon dioxide [50]. Considering the higher head concentration of hydrogen and carbon dioxide in the yeast groups, these microorganisms can utilize them to growth indicating the shaped microbial structure can utilize multiple substrates to increase the microalgae utilization rate. Also, some genus which can directly utilized microalgae or yeast cells were increased such as *Anaerolinea* (0 to 0.02 %) [51,52]. The increase of this Genus was attributed to the increased microalgae utilization rate in the yeast group.

In addition, the introduction of yeast into the fermentation process also led to a reduction in competition among certain microorganisms. For instance, the genus *Casimicrobium*, known for its ability to grow on mono- and disaccharides as well as short-chain fatty acids [53], exhibited a lower relative abundance in the yeast-supplemented group (0.8 %) compared to the control group (1.2 %). Furthermore, the genus *Candidatus Competibacter*, which harbors genes involved in glycogen and polyhydroxyalkanoate (PHA) cycling and the metabolism of volatile fatty acids [54], also demonstrated a lower abundance in the yeast group. This reduction in the abundance of *Candidatus Competibacter*, along with *Casimicrobium*, implies that the addition of yeast creates an environment less favorable for these competing microorganisms. These

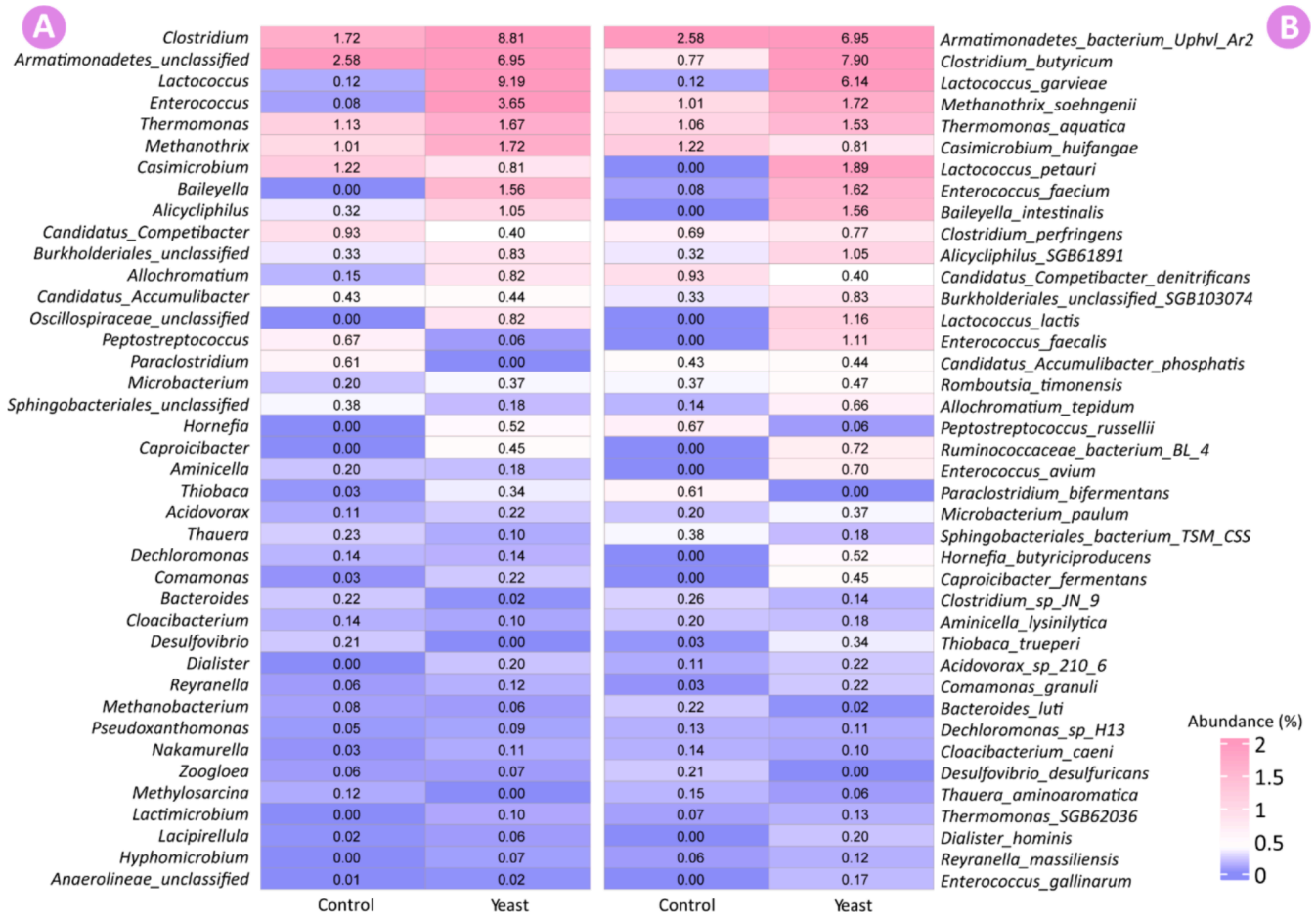


**Fig. 3.** The heatmap of microbial structure in the control and yeast group under (A) Genus and (B) Species level. The abundance indicated in the heatmap was obtained by using MetaPhlAn 4 from trimmed reads as input data. All unassigned taxa were deleted but were still contained in the total abundance.

findings indicate that yeast supplementation not only enhances substrate utilization but also increases the competitiveness of chain elongating microorganisms. By reducing the presence of competing microbes like *Casimicrobium* and *Candidatus Competibacter*, the yeast helps to direct the metabolic potential of the microbial community more efficiently towards the desired chain elongation processes, thereby improving the overall performance and efficiency of the fermentation system.

### 3.3. The metabolic potentials in this bioaugmentation process

#### 3.3.1. Metabolic potentials to utilize complex carbohydrates

Carbohydrates were the most attractive substances in our study, as they accounted for a large proportion of the microalgae COD (~17.5 %) and was verified to sufficiently convert to MCCA in the previous research. Therefore, to investigate the substrate utilization performance, the CAZyme database was used to analyze the microbial metabolic potentials to degrade complex carbohydrates. The glycoside hydrolases (GHs) class was of particular interest in our study because they can release sugars that can be easily metabolized by microorganisms as well as yeast that do not express complex carbohydrate-degrading enzymes. The results showed that the yeast group has a better carbohydrates hydrolysis potential due to a higher abundance of GHs except for GH13 (Fig. 4A, Table S1). This result indicated that the added yeast increased the carbohydrates hydrolysis potential, thereby enhancing the acid production performance.

#### 3.3.2. Metabolic potentials for chain elongation

In addition, the microbiome gene abundance data was analyzed to predict the chain elongation potential between control and yeast groups. Fig. 4B and Table S2 show the whole acyl-related gene for chain elongation. The results indicated that the addition of yeast significantly increased some of the key genes including cetyl-CoA C-acyltransferase (ACAT), acyl-CoA dehydrogenase (ACD) and alcohol dehydrogenase (ADH). These genes were responsible for the conversion of SCCA and ethanol to the corresponding acyl-CoA (Fig. 5). Acetyl-CoA is an essential intermediate product in the rBOX process of acetate and ethanol. The conversion of acetate to acetyl-CoA can through two pathways, 1) a one-step pathway involving ATP-dependent acetyl-CoA synthase (ACS) or 2) a three-step pathway involving acetate kinase (ACK), phosphate acetyltransferase (PTA) and CoA transferase (CoAT) [55]. Our study revealed a higher abundance of these genes in the yeast group compared to the control group. The lack of statistical significance in the obtained results could be ascribed to the relatively brief cultivation period (29 days) in the batch experiments. This limited timeframe may not have allowed for substantial substrate utilization, resulting in an insufficient shaping of the microbial structure, especially when compared to continuous reactor conditions. To holistically explore the influence of yeast addition on the chain elongation process, it is imperative to conduct metagenomic and metatranscriptome analyses within a continuous reactor setup. This approach will provide a more in-depth understanding, as the continuous reactor model offers a more stable environment for microbial interactions and metabolic processes.

In both the control and yeast groups, a small number of genes involved in ferredoxin hydrogenase production were detected,

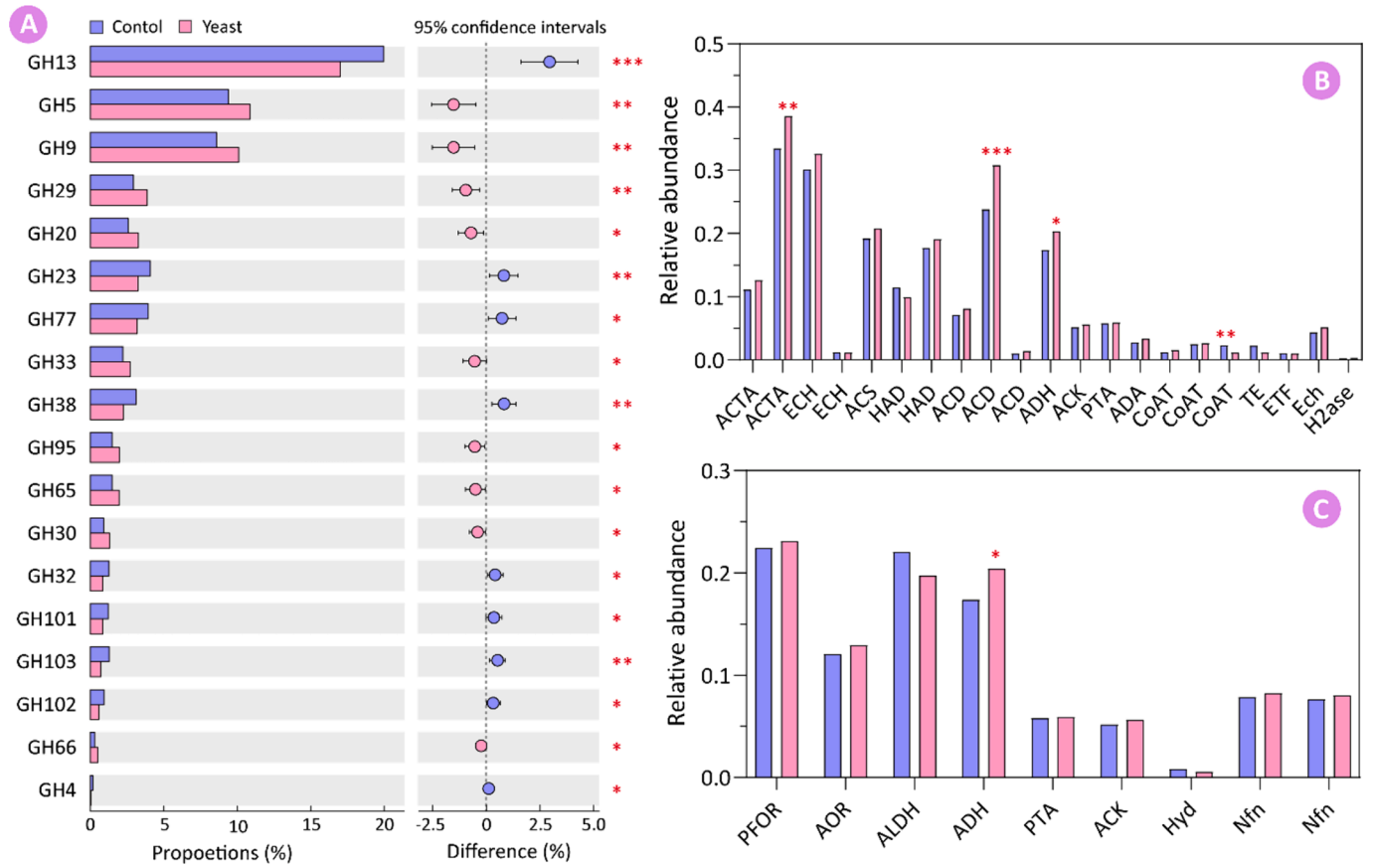


**Fig. 4.** The relative abundance of (A) glycoside hydrolase family (The bar plots indicate the proposition (%) of corresponding genes in the whole sample, while the differences between two group are showed in the scatter plots), (B) acyl chain elongation genes and (C) butanol production genes (exact Fisher's test: * $0.01 \leq P \leq 0.05$, **$0.001 < P \leq 0.01$ ***:$P \leq 0.001$).

providing support for the hypothesis that $H_2$ production contributes to the MCCA producers [56]. Additionally, we investigated the presence of two other hydrogenases, EchABCDEF and HydABC, which are known to generate energy through proton translocation or electron confurcation using nicotinamide adenine dinucleotide hydrogen (NADH) and reduced ferredoxin, respectively [57,58]. These genes encoding the known components of these enzyme complexes, indicating that these systems likely play a significant role in $H_2$ production in these microbiomes (Fig. 4B and 4C, Table S2).

### 3.3.3. *Metabolic potentials for butanol production*

Two distinct pathways are responsible for the butanol production from butyryl-CoA as illustrated in Fig. 5 and Table S2 [46,59]. In the direct pathway, butyryl-CoA is enzymatically converted to butyraldehyde and subsequently reduced to butanol by a bifunctional alcohol dehydrogenase (ADH) [60]. On the other hand, the indirect pathway involves the initial conversion of butyryl-CoA to butyrate, with the intermediate butyryl phosphate (butyryl-P) formed through the action of PTA. The subsequent step involves the transformation from butyryl-P to butyrate by ACK, leading to the production of ATP via substrate-level phosphorylation [61]. Undissociated butyric acid is reduced by an aldehyde:ferredoxin oxidoreductase (AOR) using electrons from reduced ferredoxin (Fdred), leading to the formation of butyraldehyde [62]. The changes in the relative abundance of these genes are depicted in Fig. 4C. The results demonstrate that the addition of yeast significantly increased the abundance of ADH genes from 0.17 % to 0.20 %. The yeast group showed a decrease in ALDH abundance and a slight increase in AOR genes, suggesting that the addition of yeast altered the metabolic potential of the microbiome towards the indirect pathway for butanol production. Furthermore, the higher concentrations of acetate and butyrate in the yeast group indicate a potential increase in AOR activity compared to the control group. In prior studies, it has been documented that the introduction of exogenous acetate can induce a further shift in the product composition towards alcohol in specific acetogens [61,63]. Richter et al. (2016) provided evidence indicating that the production of alcohol is governed by thermodynamic principles rather than transcriptional or translational regulation. Although their model was initially proposed to elucidate the factors influencing ethanol production, its applicability extends to our findings of enhanced butanol production, considering the involvement of similar enzyme systems [64].

## 4. Discussion

This study presents pioneering evidence of yeast significantly enhancing the production of carboxylates and butanol from microalgae biomass. The addition of yeast resulted in a fourfold increase in total carboxylate concentration, reaching 184.6 ± 16.2 mM-C compared to the control group. Given the absence of ethanol production in the control group, the enhanced performance can be entirely attributed to the *in-situ* production of ethanol by yeast. This ethanol not only acted as an electron donor for chain elongation but also served as a reducing force for butyrate reduction, elucidating the observed enhanced performance in the experimental group. This synergistic action effectively prolonged the carbon chain of fermentation products into MCCAs with a total concentration of 28.0 ± 3.4 mM-C and butanol with a concentration of 1.9 ± 0.2 mM-C. Yeast plays a critical role by fermenting the sugars derived from microalgae into ethanol, which serves as an essential electron donor in the chain elongation process conducted by bacteria. The chain-elongating bacteria utilize this ethanol along with acetate and

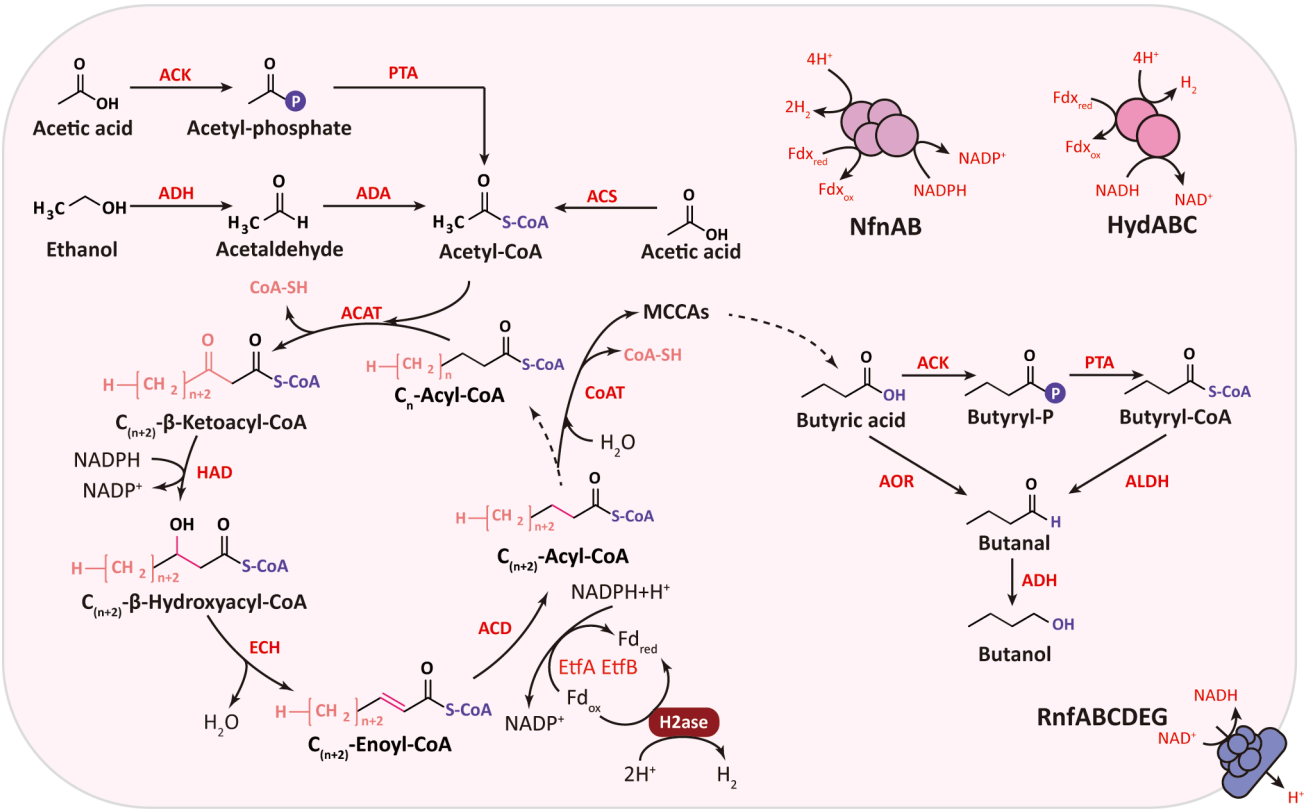


**Fig. 5.** Proposed microbial pathway for MCCA and butanol production. Acetaldehyde dehydrogenase (ADA, EC 1.2.1.10), alcohol dehydrogenase (ADH, EC 1.1.1.1), phosphate acetyltransferase (PTA, EC 2.3.1.8), acetate kinase (ACK, EC 2.7.2.1), acetyl-CoA synthetase (ACS, EC 6.2.1.1), acetyl-CoA C-acyltransferase (ACAT, EC 2.3.1.16, EC 2.3.1.9), 3-hydroxyacyl-CoA dehydrogenase (HAD, EC 1.1.1.157, 1.1.1.35), enoyl-CoA hydratase (ECH, EC 4.2.1.55, EC 4.2.1.17), acyl-CoA dehydrogenase (ACD, EC 1.3.8.1, EC 1.3.8.7, EC 1.3.8.8), electron transfer flavoprotein (Etf, EC 1.5.5.1), thioesterase (TE, EC 3.1.2.20), 4-hydroxybutyrate CoA transferase (CoAT EC 2.8.3.1, EC 2.8.3.8, EC 2.8.3.9), ferredoxin–$NAD^+$ oxidoreductase–$Na^+$ translocating (Rnf, EC 7.2.1.2), ferredoxin hydrogenase (H2ase, EC 1.12.7.2), bifurcating [Fe-Fe] hydorgenase (HydABC, EC 1.12.1.4), energy-conserving hydrogenase (EchABCDEF, EC 1.12.2.1), pyruvate ferredoxin oxidoreductase (PFOR, 1.2.7.1), aldehyde ferredoxin oxidoreductase (AOR, EC 1.2.7.5), aldehyde dehydrogenase (ALDH, EC 1.2.1.3), NADH-dependent reduced ferredoxin:NADP oxidoreductase (Nfn, EC 1.18.1.2, EC 1.19.1.1).

butyrate, which are typical short-chain carboxylates, to synthesize longer-chain carboxylates through a metabolic pathway known as reverse β-oxidation. This interaction not only increases the efficiency of substrate utilization, converting a larger portion of microalgae-derived carbohydrates into valuable carboxylates but it also enhances the stability and robustness of the microbial community.

Previous research has delved deeply into the influence of ethanol on chain elongation and alcohol synthesis. For example, Wu et al. achieved an MCCA yield of approximately 19.8 mM-C/g VS by utilizing waste activated sludge alkaline fermentation liquid as a substrate with the addition of 226.6 mM ethanol [65]. Similarly, Wang et al. obtained a caproic acid yield of 53.6 mM-C/g VS by using 651.0 mM ethanol and anaerobic fermentation liquid of sewage sludge as substrates [66]. In our study, we achieved an MCCA yield of 46.3 mM-C/g VS without the addition of any extra ethanol, demonstrating the feasibility of this novel process for MCCA production. It is imperative to note that equivalent MCCA yields do not necessarily indicate identical fermentation efficiencies. Elevated ethanol concentrations could potentially instigate excessive ethanol oxidation, subsequently providing an abundance of electron acceptors for chain elongation [67]. The ethanol was first oxidized to acetaldehyde by alcohol dehydrogenase. Acetaldehyde is further oxidized to acetate via acetaldehyde dehydrogenase. The higher abundance of ADH in yeast group verified this process. Consequently, the high MCCA yield might predominantly originate from ethanol rather than biowastes. In our methodology, external ethanol supplementation was circumvented, with the requisite ethanol for chain elongation being intrinsically generated from microalgae by the yeast. This resulted in an augmented substrate utilization efficiency, bolstering the economic viability of the yeast-mediated chain elongation system.

In addition, the accumulation of acetate suggests a slightly inhibition in chain elongation, likely attributable to the weak acidic conditions present. The COD fraction of SCCAs among total fermentation derivatives, representing approximately 70 % (Fig. 1E), points to suboptimal chain elongation efficacy. A mildly acidic environment is advocated to suppress the methanogenic pathway, thereby minimizing the need for methanogenic inhibitors [68]. This diminished efficacy can be ascribed to the pH conditions, specifically those below pH 5, which predominantly favor ethanol synthesis through yeast fermentation [19]. The pH plays a pivotal role in determining the primary products in the chain elongation system. Studies have demonstrated that neutral pH conditions are conducive to MCCA synthesis [69,70]. Nonetheless, A salient limitation of acidic environments in the chain elongation system is the pronounced product toxicity to the chain elongator, even at minimal MCCA concentrations. In our study, we found that both chain elongator and yeast have a relatively high activity at pH 5, verifying the feasibility of this novel process. However, subsequent research could enhance MCCA production rates by segregating the yeast fermentation and chain elongation processes, operating them under distinct pH regimes.

The enhanced performance can be attributed to the growth of chain elongators (*Clostridium*). The utilization of gases by some species also increased the substrate utilization rate. The addition of yeast directed the conversion of reducing sugars into ethanol, facilitating MCCA production through chain elongation. Moreover, the production of carbon

dioxide from yeast metabolism increased the proportion of *Thermomonas* and *Methanothrix* which is known to reduce carbon dioxide through hydrogen [49,50]. This enhanced gas utilization further increased the carbon utilization rate in the yeast-induced chain elongation system. Metagenomic analysis supported these observations, showing increased abundance of genes related to acetyl-CoA production in the yeast group, while genes associated with the utilization of complex substrates were more abundant in the control group. This metabolic shift redirected the carbon and electron flow towards the production of MCCAs and butanol rather than biomass increase. In addition, the taxonomic origins analysis indicated that the most common species involved in the rBOX pathway belonged to the phyla *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (Tables S3-S29). Although previous research identified various *Firmicutes* capable of chain elongation, few were associated with *Proteobacteria* and *Actinobacteria* [71]. Our study suggested that these phyla might also produce MCCAs through the rBOX pathway due to the presence of relevant genes. However, metatranscriptomic analysis should be conducted to provide further evidence.

The addition of yeast also induced the production of butanol. Metagenomic evidence suggested the significant role of the AOR pathway in butanol production. This pathway utilizes an Fd-dependent reaction to reduce organic acids to aldehydes, which are then further reduced to alcohols by NAD(P)H-dependent alcohol dehydrogenase (ADH). Several studies have indicated that the AOR pathway is the primary route for ethanol and butanol production in autotrophic or sugar-based fermentation by acetogens such as *C. ljungdahlii* and *C. ragsdalei*. Species like *C. saccharoperbutylacetonicum* and *C. thermoanaerobacter* species with AOR and heterologous alcohol dehydrogenase (*adh*) genes can produce ethanol from glucose and convert exogenous carboxylic acids into their corresponding alcohol [72,73]. For example, Richter et al. achieved a butanol yield of 0.4 g/(L·h) by reducing supplemented butyrate in continuous cultures of a *C. saccharoperbutylacetonicum* reactor [74]. Our metagenomic data indicated that most AOR genes were found in *Proteobacteria* and *Chloroflexi*, except for *Clostridium* (Table S9), suggesting more metabolic diversity than previously reported. The strong correlation between butyrate concentration and AOR abundance indicated the significant role of the AOR pathway in butanol production (Fig. 1A and Fig. 4C). The high concentration of organic acids could accelerate the regeneration of reducing equivalents, leading to faster metabolism and increased growth rates [75]. Additionally, higher hydrogen supply in the yeast group resulted in a higher nicotinamide adenine dinucleotide ($NADH/NAD^+$) ratio, thereby increasing the rate of organic acid reduction. However, it remains to be determined to what extent AOR, ALDH, and alcohol dehydrogenases contribute to the reduction of organic acids to their corresponding alcohols.

This work aimed to verify the feasibility of yeast in promoting MCCA production. In real applications, simply adjusting the pH to 5 and maintaining a temperature of 35 °C can facilitate anaerobic microalgae fermentation to produce medium-chain carboxylic acids without the need for additional controls. However, a notable limitation was observed in the accumulation of acetate and butyrate during the demonstration phase, primarily due to the acidic conditions that inhibit chain elongation. Therefore, separating yeast fermentation and chain elongation into two reactors can enhance the production rate of MCCAs. Additionally, the optimization of yeast dosage can further reduce operational costs and increase the production rate of the desired products. Regarding economic feasibility, previous research has developed the yeast encapsulation process to immobilize the yeast within the bioreactor [76,77], thereby reducing the cost of yeast supplementation in continuous anaerobic microalgae fermentation. In summary, the integration of yeast fermentation and microalgae fermentation demonstrates the feasibility in resource recovery from waste microalgae biomass.

## 5. Conclusion

This research proposed a novel integrated process that involves in-situ production of both electron donor and acceptor from microalgae through bioaugmentation of microalgae fermentation with yeast. The results revealed that the addition of yeast led to a fourfold increase in carboxylate yield. This novel process elongates the carbon chain of fermentation products, resulting in the synthesis of medium-chain carboxylic acids with a yield of 46.3 mM-C/g VS. This enhancement was not solely due to the in-situ production of ethanol but also to the high substrate utilization rate in the yeast group. The microbial structure unveiled the pivotal role of heightened chain elongator presence in this enhancement. Moreover, the organized microbial guild emerged as a key factor in enhancing substrate utilization rates. Metabolic potential analysis underscored the increased abundance of genes linked to acetyl-CoA production as the primary pathway for MCCA synthesis, while the AOR-related pathway orchestrated butanol production. In essence, this research pioneers a revolutionary chain elongation process for the production of MCCAs and butanol, achieving this milestone without reliance on external electron donors.

## CRediT authorship contribution statement

**Xingdong Shi:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Feng Ju:** Writing – review & editing, Methodology, Formal analysis. **Wei Wei:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis. **Lan Wu:** Writing – review & editing, Methodology. **Xueming Chen:** Writing – review & editing, Methodology, Formal analysis. **Bing-Jie Ni:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.155742.

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