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Enhancing bacterial cellulose production of *Komagataeibacter nataicola* through fermented coconut water by *Saccharomyces cerevisiae*: A metabonomics approach

Shuangwen Fei [a,1], Meijuan Fu [a,1], Jiamu Kang [a], Jiaxi Luo [a], Yanmei Wang [a], Jia Jia [a], Sixin Liu [a,b,c,*], Congfa Li [a,b,c,**]

[a] School of Food Science and Engineering, Hainan University, Haikou, 570228, China
[b] Key Laboratory of Food Nutrition and Functional Food of Hainan Province, Haikou, 570228, China
[c] Key Laboratory of Tropical Agricultural Products Processing Technology of Haikou, Haikou, 570228, China

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ABSTRACT

Nata de coco, an edible bacterial cellulose (BC) product, is a traditional dessert fermented in coconut water. Production of *Nata de coco* by *Komagataeibacter nataicola* is enhanced by pre-fermented coconut water, but its instability is a challenge. Here, BC production by *K. nataicola* Y19 was significantly improved by *Saccharomyces cerevisiae* 84-3 through shaping the metabolite profile of the coconut water. Different fermentation time with *S. cerevisiae* 84-3 resulted in distinct metabolite profiles and different promoting effect on BC yield. Compared to unfermented coconut water, coconut water fermented by *S. cerevisiae* 84-3 for 1d and 7d enhanced BC yield by 14.1-fold and 5.63-fold, respectively. Analysis between unfermented coconut water and 1d-fermented coconut water showed 129 significantly different metabolites, including organic acids, amino acids, nucleotides, and their derivatives. Prolonged fermentation for 7d changed levels of 155 metabolites belongs to organic acids, amino acids, nucleotides and their derivatives. Spearman correlation analysis further revealed that 17 metabolites were positively correlated with BC yield and 21 metabolites were negatively correlated with BC yield. These metabolites may affect energy metabolism, cell signaling, membrane integrity, and BC production by *K. nataicola* Y19. The further verification experiment gave the view that BC yield was not only closely related to the types of metabolites but also the concentration of metabolites. This study provides a novel theoretical framework for a highly efficient BC fermentation system utilizing stable fermented coconut water mediums.

1. Introduction

Coconut water is the liquid endosperma of coconut and has various nutrient substances including sugar, amino acids, vitamins, and minerals which makes coconut water a natural medium for microorganism fermentation (Xu et al., 2022). *Nata de coco* is a globally popular dietary fiber resource and a traditional diet food with low calories, unique texture, and delicious taste. *Nata de coco* is produced in coconut water and is widely used as an ingredient in various beverages and desserts in China and Southeast Asian countries (Ullah, 2016). The primary component of *Nata de coco* is BC, a kind of microbial extracellular polysaccharide with high purity, high tensile strength, and good biocompatibility, which makes BC straightforward to process and pollution-free, presenting significant potential for applications such as biodegradable food packaging bags and Pickering emulsions (Cazon, 2021). Thus, the production improvement of nata de coco i.e. BC is crucial and have been widely focused during the past decades (Ul-Islam et al., 2020).

In China and Southeast Asian countries, natural fermented coconut water was widely used for *Nata de coco* production. However, natural fermentation is unstable and uncontrollable, which is a big change for high-efficient BC production (Zhang et al., 2017). In our previous study, *Saccharomyces* was identified as the dominant fungus in natural fermented coconut water (Qin et al., 2024). In the following exploration,

* Corresponding author. School of Food Science and Engineering, Hainan University, No. 58 Renmin Ave., Haikou, 570228, China.
** Corresponding author. School of Food Science and Engineering, Hainan University, No. 58 Renmin Ave., Haikou, 570228, China.
E-mail addresses: sixinliu@hainanu.edu.cn (S. Liu), congfa@hainanu.edu.cn (C. Li).
[1] Co-first author.

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we isolated a strain of *Saccharomyces cerevisiae* 84-3 from naturally fermented coconut water and preliminary confirmed that coconut water fermented by *S. cerevisiae* 84-3 could significantly increase BC yield. Furthermore, we also found that fermentation time was an essential factor that affected BC yield. However, the underlying mechanism is still unclear and has not been investigated. As it is known to all, fermentation is a traditional processing technology that changes the food composition. *S. cerevisiae*, a famous yeast, has been widely used for the fermentation (Gallone et al., 2016) and its fermentation could change the metabolites profile of foods (Zhang et al., 2018). We proposed the hypothesis that the increased BC yield were caused by key changed metabolites during *S. cerevisiae* 84-3 fermentation in coconut water.

Metabolomics is a comprehensive study that qualitatively, quantitatively, and dynamically analyzes small molecules ($\leq$1 kDa) within biological fluids, cells, and organisms. It is a powerful tool that can unveil the molecular mechanisms underlying various phenomena (Wang et al., 2021). Currently, metabolomics methods are widely used to investigate the changes in the metabolic spectrum of coconut water under varying conditions and elucidate their underlying mechanisms. For instance, Chen et al. (2018) utilized UPLC-MS/MS to investigate the changes in the metabolome of coconut water during the transportation process after coconut maturation, determining the optimal preservation time after coconut harvest and identifying twelve biomarkers of coconut water with good taste. Cunha et al. (2020) employed UPLC-HRMS to investigate the effects of heat treatment on metabolites in coconut water. In addition, untargeted metabolomics methods have gained attention for their wide detection range and strong identification capabilities (Utpott et al., 2021). Thus, this technology can serve as an effective tool for elucidating the metabolomic changes responsible for *S. cerevisiae* fermentation in coconut water.

The genera capable of fermenting to producing BC mainly include *Komagataeibacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Burkholderia* (Moradi et al., 2021). Among these, *Komagataeibacter* is widely used in researches and industry due to its higher and stable BC yield (Moradi et al., 2021). Thus, a *K. nataicola* strain with stable BC yield was used in this present study. In this study, the BC yield of *K. nataicola* Y19 in the coconut water fermented by *S. cerevisiae* 84-3 was compared across different fermentation days. Meanwhile, the physicochemical properties of coconut water fermented by *S. cerevisiae* 84-3 at different fermentation times were assessed. Further, untargeted metabolomics based on Ultra-performance Liquid Chromatography Combined with Mass Spectrometry (UPLC-ESI-MS/MS) was utilized to examine the metabolic profile changes in coconut water on different fermentation days of *S. cerevisiae* 84-3. Spearman correlation was used to screen the potential metabolite that positively related with BC yield. Exogenous addition experiments were performed to verify the hypothesis.

2. Materials and methods

2.1. Materials

Fresh coconuts were purchased from the local market in Haikou, China. *K. nataicola* Y19 was preserved in the School of Food Science and Engineering, Hainan University (Bi et al., 2014). *S. cerevisiae* 84-3, an efficient strain isolated from the natural fermented coconut water provided by the local *Nata de coco* production factory, has been used for BC yield improvement by fermenting coconut water in our laboratory. This strain was identified by the Beijing Genomics Institution (Beijing, China). The primer used in here is ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The identified results and annotation of *S. cerevisiae* 84-3 were provided in Supplementary Data 1.

2.2. Fermentation of coconut water

Fresh coconuts were cracked in the laboratory to obtain coconut water, which was subsequently filtered through three layers of sterile gauze and collected in sterile plastic bottles for further research. Following that, *S. cerevisiae* 84-3 was inoculated into the coconut water at an inoculum size of 2%(v/v) and statically fermented at 30 °C in incubator (Yiheng Scientific Instrument Co., Shanghai, China). Samples were collected every 24 h during the fermentation of 7d. Unfermented coconut water was used as a control and abbreviated as F0. All unfermented/fermented coconut water were temporarily frozen at −20 °C until use.

2.3. Physicochemical properties analysis

The pH values and total soluble solids (°Bx) of the unfermented/fermented coconut water were measured using a pH meter (METTLER TOLEDO, Zurich, Switzerland) and a refractometer (PAL-1, Atago, Tokyo, Japan), respectively. The total titratable acidity was determined following the procedure of acid-base titration method in China National Standard (GB/T 12456-2008). Reducing sugar was tested according to the direct titration method in China National Standard (GB 5009.7-2016). The ethanol content was measured following the third method in China National Standard (GBT15038-2006). The concentrations of acetic acid, lactic acid, malic acid, pyruvic acid, and gluconic acid in each group were determined using a high-performance liquid chromatograph with a VWD detector (HPLC-2030, Shimadzu, Japan). Every sample was filtered through a 0.22 μm water membrane and collected for subsequent analysis. The mobile phase consisted of 0.1% perchloric acid and methanol (92:2, v/v) at a flow rate of 0.6 mL/min. The quantification and identification of the above organic acids were performed based on the standard curves and retention times of the corresponding standards (Solarbio, Beijing, China).

2.4. Effect of fermented coconut water on BC yield

The *K. nataicola* Y19 was activated and then inoculated at a concentration of 2% (v/v) into a 100 mL flask containing 50 mL of unfermented/fermented coconut water-based medium. The medium consisted of 0.15g $(NH4)_2SO_4$, 0.015g KH_2 PO_4, 0.015g $MgSO_4{\cdot}7H_2O$, 50% (v/v) unfermented/fermented coconut water, 50% (v/v) distilled water, and had a pH of 5.5. Subsequently, unfermented/fermented coconut water was sterilized at 115 °C for 20 min to prevent microbial interactions during BC fermentation. The inoculated flask was then incubated at 30 °C under undisturbed static conditions in biochemical incubator (Yiheng Scientific Instrument Co., Shanghai, China). The BC film was harvested after 7 d and washed for three times with distilled water to remove surface dirt. BC films were immersed in 0.1 mol/L NaOH solution and then warmed at 60 °C for 24h.The alkali-treated BC film was neutralized and washed with a 0.5% (v/v) acetic acid solution, followed by deionized water rinsing until the pH reached neutrality. The film was subsequently dried at 60 °C until a constant weight was achieved (Tian et al., 2018). The yield of BC was calculated in g/L based on its dry weight.

2.5. Metabolomics analysis

2.5.1. Sample preparation

F0 and fermented coconut water for 1d (F1) and 7d (F7) were freeze-dried to constant weight in a vacuum freeze dryer (Scienz-100F, Svientz, China), and then ground for 1.5 min at a frequency of 30 Hz using a grinder (MM400, Retsch, Germany). 100 mg powder of each group was placed in a 1.5 mL centrifuge tube, and then 1.2 mL of 70% (v/v) MS-grade methanol was added. The mixture was vortexed for 6 min, followed by centrifugation at 10000×*g*, 4 °C for 10 min. The supernatant was filtered through a 0.22 μm membrane and collected for subsequent analysis. Ten microliters of each unfermented/fermented coconut water sample were mixed to serve as a quality control (QC) sample. Three random QC samples were injected during the detection process to evaluate the stability of the chromatography-mass spectrometry system

and the repeatability and reliability of the experimental data.

2.5.2. UPLC-ESI-MS/MS conditions

Chromatographic separation of all unfermented/fermented coconut water samples was performed on a UHPLC (Shim-pack UFLC ShimadZU CBM30A system, Shimadzu, Japan) equipped with a Waters ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm). The eluent A was ultrapure water containing 0.04% (v/v) acetic acid, and the eluent B was MS-grade acetonitrile containing 0.04% (v/v) acetic acid. The column temperature was set at 40 °C, the injection volume was 2 μL, and the flow rate was 0.35 mL/min. The gradient program was as follows: 0–1 min, 5% B; 1–10 min, 5–95% B; 10–11 min, 95% B; 11–11.1 min, 95-5% B; 11.1–14 min, 5% B.

The mass spectrometry method was established based on the research of Chen et al. (2013) with minor modifications. In this study, an API 6500 Q TRAP UPLC/MS/MS system (Shimadzu, Japan) equipped with an ESI Turbo IonSpray interface was used. Quantitative Analysis of metabolites was performed according to the research by Ning et al. (2022) The annotation of metabolites was performed based on the Metware database (Metware Biotechnology Co., Ltd, Wuhan, Hubei, China). The system was operated in both positive and negative ion modes and was controlled by Analyst 1.6.3 software (Sciex, Framingham, USA). The ESI source temperature was set at 550 °C, and the ion spray voltage (IS) was 5500 V (positive ion mode) or −4500 V (negative ion mode). The ion source gases I (GSI), II (GSII), and curtain gas were set to 50, 60, and 30.0 psi, respectively.

2.6. Effect of metabolites addition on BC yield

Ethanol, lactic acid, and acetic acid at concentrations of 0.25% (v/v), 0.5% (v/v), 1% (v/v), 1.5% (v/v), and 2% (v/v), and 2-Indolecarboxylic acid and serine at concentrations of 0.25% (v/v), 0.5% (v/v), 1% (v/v) were added separately to the F0 (containing 0.15 g $(NH4)_2SO_4$, 0.015 g KH_2PO_4, 0.015 g $MgSO_4{\cdot}7H_2O$, 50% (v/v)) to investigate their effects on BC yield. The fermentation process and BC film collection were followed the same procedures in section 2.4.

2.7. Statistic analysis

Variable importance in projection (VIP) values fold changes (FC), and *p*-values were used as parameters to screen significantly different metabolites among different sample groups. 'MetaboAnalystR', 'prcomp', 'tidyverse' and 'pheatmap' package in R software were used for metabolites profile analysis and data visualization. Metabolites showing significant regulation between groups were identified based on VIP ≥1 and absolute $Log_2FC \geq 1$. Cytoscape (3.3.0) was used to visualize the correlation between metabolites and BC yield. The normality of all data was assessed using SPSS (version 26). Each sample was measured in triplicate, and the data is presented as mean ± standard deviation.

3. Results

3.1. Effect of S. cerevisiae fermentation on BC yield in coconut water

The BC yield of *K. nataicola* Y19 in coconut water fermented by *S. cerevisiae* was evaluated (Fig. 1A). The BC yield was the lowest in F0 (0.50 ± 0.06 g/L) and increased by 14.1 times (7.07 ± 0.03 g/L) in F1. With the increase of fermentation time of coconut water, the BC yield was decreased obviously. F7 just promoted the BC yield by 5.36 times (2.68 ± 0.37 g/L).

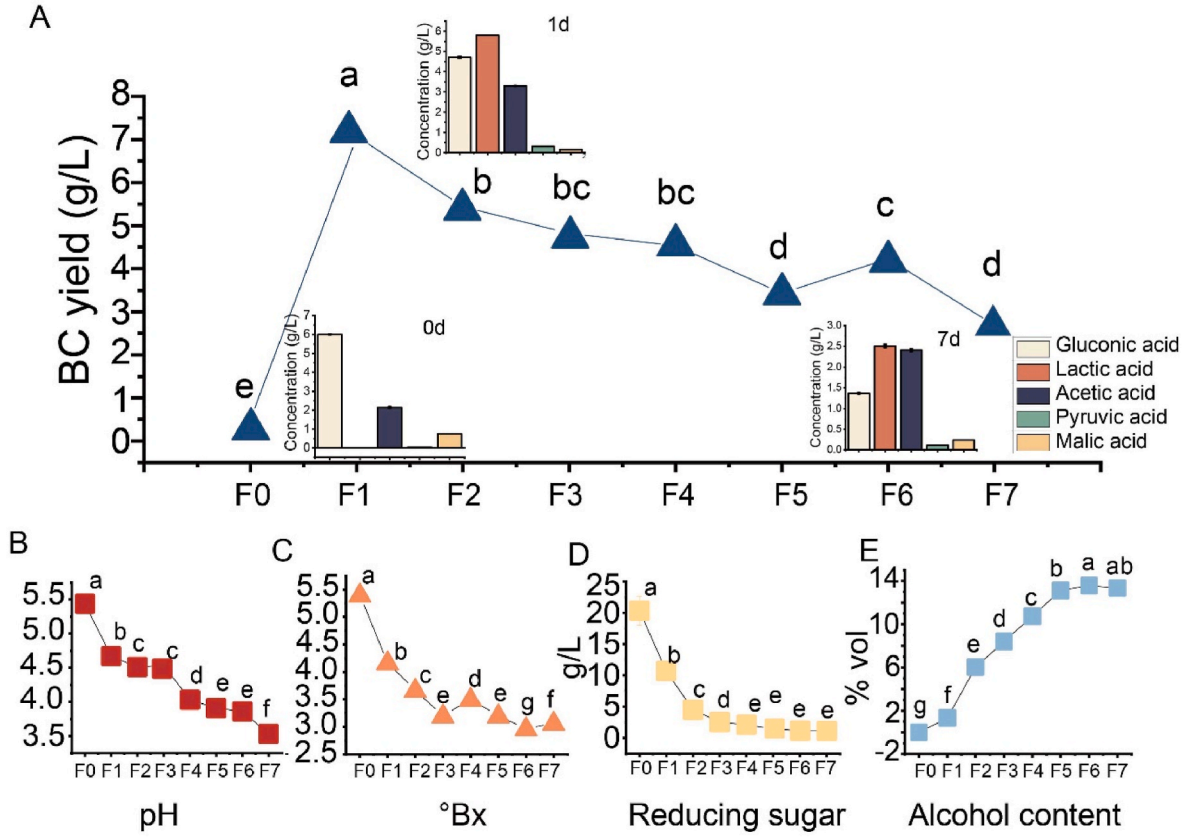

Fig. 1. Physicochemical characteristics of *S. cerevisiae* 84-3 fermented coconut water and its effects on BC yield. F0–F7 are abbreviations of coconut water fermented by *S. cerevisiae* 84-3 for 1d to 7d. Carnatio represents gluconic acid, orange represents lactic acid, blue represents acetic acid, green represents pyruvic acid, yellow represents malic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Physicochemical characteristics of coconut water fermented by S. cerevisiae

As shown in Fig. 1, the fermentation time of *S. cerevisiae* 84-3 led to significant changes in the pH value and organic acid components of the coconut water. The pH of coconut water gradually decreased from 5.5 ± 0.00 to 3.5 ± 0.01 after fermentation of *S. cerevisiae* 84-3 from 1d to 7d. Furthermore, with the increasing fermentation time, sugar content and reducing sugar content of the coconut water decreased from 5.4 °Bx and 20 ± 1.61 g/L to 3.6 ± 0.05 °Bx and 1.15 ± 0.04 g/L, respectively. The ethanol content of coconut water fermented by *S. cerevisiae* 84-3 was 1.33 ± 0.06%vol, then it increased dramatically to 13.31 ± 0.09 %vol at the end of *S. cerevisiae* 84-3 fermentation ($p < 0.05$). Additionally, the concentration of gluconic acid in F0 was 6.01 ± 0.02 g/L and decreased to 4.71 ± 0.05 g/L and 1.37 ± 0.02 g/L in F1 and F7, respectively. The levels of lactic acid and acetic acid in F1 were significantly higher than those in F0 and F7. The pyruvic acid content in the coconut water did not significantly change during the *S. cerevisiae* 84-3 fermentation processes. As the pre-fermentation of *S. cerevisiae* 84-3 progressed, the malic acid content decreased from 0.75 ± 0.00 g/L (F0) to 0.16 ± 0.00 g/L (F1) and then increased to 0.24 ± 0.00 g/L (F7).

3.3. Metabolomics analysis

3.3.1. Construction of metabolomics model

To explore the metabolic profiles of fermented coconut water, an untargeted metabolomics analysis using UPLC-ESI-MS/MS was conducted. The peak area was used to calculate the correlation coefficient between the three QC samples, resulting in an R^2 value of ≥0.972 for each sample. This suggested that the metabolomics results were highly repeatable and reliable. The abundance values of each metabolite were normalized using unit variance scaling, and a heatmap (Fig. 2A) was constructed to visualize the differences between groups. Following this, a PCA model was constructed for the metabolomics data to determine the classification of metabolites in F0, F1, and F7. As depicted in Fig. 2B, the QC samples were located in the central area of the tested samples, indicating excellent repeatability and stability of the data. Moreover, samples within F0, F1, and F7 clustered closely together and separated between groups, indicating high similarity of samples within the group and large differences in metabolites between different groups.

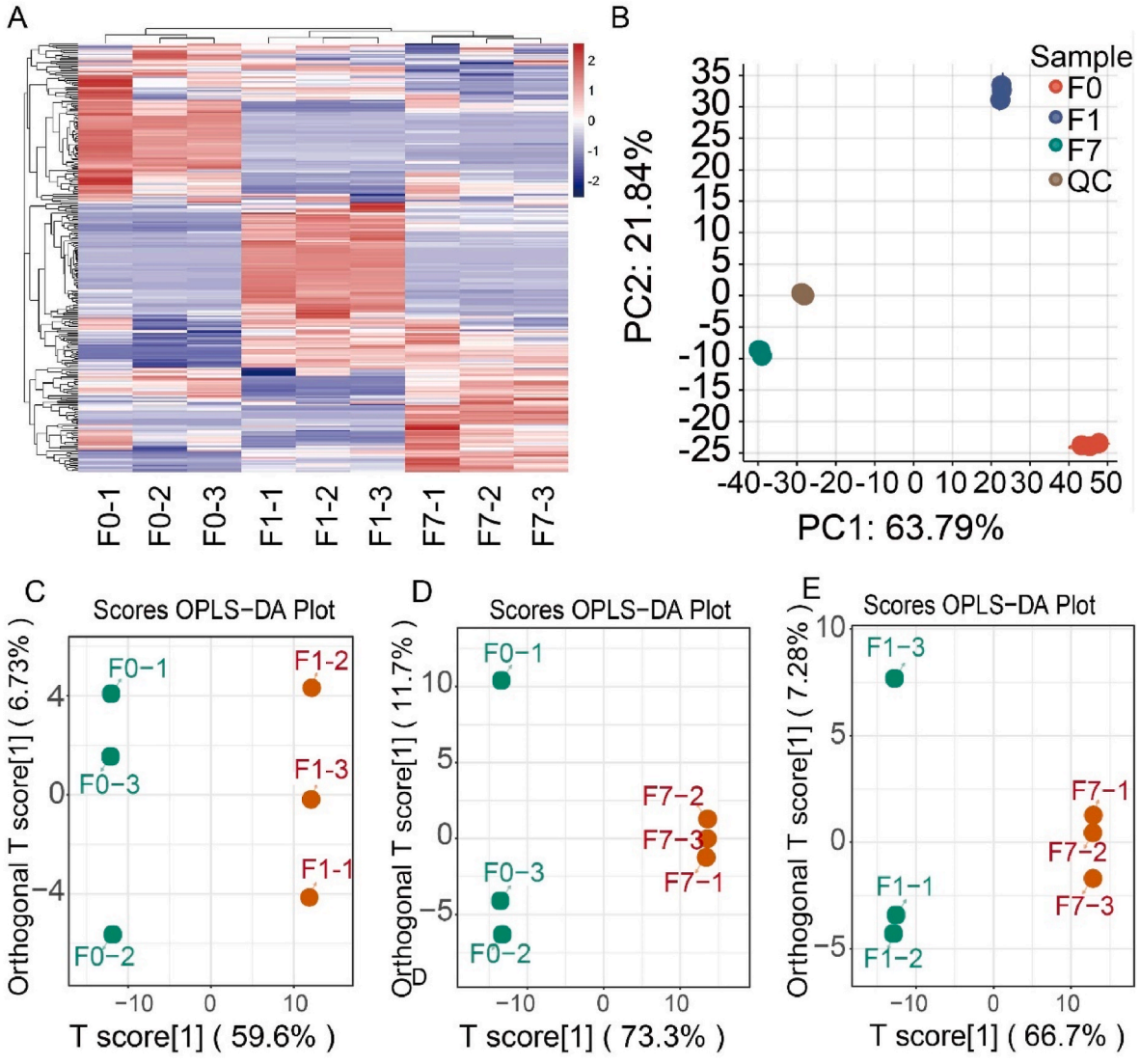

Fig. 2. Relative abundance of metabolites in unfermented coconut water (F0) and coconut water fermented by *S. cerevisiae* 84-3 for 1d (F1) and 7d (F7). (A) Heatmap of differential metabolites. Red or blue color represents the increase or decrease in content of the different metabolites. (B) PCA score plots. QC: Quality Control. Orthogonal projection to latent structure discriminant analysis (OPLS-DA) between the F0 *vs.* F1 (C), F0 *vs.* F7 (D) and F1 *vs.* F7 (E). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

To identify the differential metabolites, the data model needs to be validated first. Validation was carried out through OPLS-DA analysis on the comparisons of F0 *vs.* F1, F0 *vs.* F7, and F1 *vs.* F7 (Fig. 2C–E). Additionally, a seven-fold cross-validation was conducted to obtain model evaluation parameters, including R^2X, R^2Y, and Q^2, to assess the reliability of the OPLS-DA model. The parameters between F0 *vs.* F1 were: $R^2X = 0.664$, $R^2Y = 1$, $Q^2 = 0.966$; the parameters between F0 *vs.* F7 were: $R^2X = 0.85$, $R^2Y = 1$, $Q^2 = 0.988$; and the parameters between F1 *vs.* F7 were $R^2X = 0.739$, $R^2Y = 1$, $Q^2 = 0.98$. These findings demonstrated the stability of the results and confirmed the predictability and reliability of the established discriminant model.

3.3.2. Analysis of significantly differential metabolites

A total of 296 metabolites were identified in the three coconut water samples, including organic acids, fatty acids, feather oils, vitamins, sugar alcohols, alkaloids, nucleotides and their derivatives, resins, phenolic acids, and amino acids and their derivatives. The compositions of F0, F1, and F7 showed distinct differences, indicating that pre-fermentation significantly changed the metabolite composition in coconut water. Subsequently, a volcano plot was used to display the overall distribution of differential metabolites between groups (Fig. 3), where each point represents a metabolite. The red represents significantly upregulated metabolites (VIP≥1, $p \leq 0.05$, FC ≥ 2), and the blue represents significantly downregulated metabolites (VIP≥1, $p \leq 0.05$, FC ≤ 0.05). The analysis found that there were 129 significantly differential metabolites (DMs) between F0 and F1, mainly amino acids and their derivatives, and nucleotides and their derivatives. Among those DMs, 58 metabolites were downregulated, and 71 metabolites were upregulated (Fig. 3A). A comparison between F0 and F7 revealed 176 DMs, primarily consisting of amino acids and their derivatives, vitamins, and sugar alcohols. Of these, 90 metabolites were downregulated, while 89 were upregulated (Fig. 3B). The mevalonic acid, fumaric acid, phenylpyruvic, acid xylitol, D-arabitol, phenyllactate, postbiotics, and 12-hydroxydodecanoic acid was synthesized by *S. cerevisiae* 84-3 when fermented for 1d (Table 1). The prolonged fermentation time of *S. cerevisiae* 84-3 would lead to significant changes in amino acids and their derivatives, nucleotides and their derivatives, and organic acids. Totally, there were 155 DMs between F7 and F1, among which 65 were downregulated, and 90 were upregulated (Fig. 3C). As listed in Table 2, with the increase of fermentation time, the content of α-hydroxyisobutyric acid, 9-(β-D-arabinofuranosyl)hypoxanthine, (S)-2-hydroxybutanoicacid, L-(+)-tartaric acid, (5-L-glutamyl)-L-amino acid, N-acetylaspartate, cinnamic acid, and sebacate were largely formed while citric acid, pinoresinol, diglucoside, syringin, cyclic AMP, pinoresinol-hexose, terpineol monoglucoside, 2,5-dihydroxy benzoic acid, O-hexside, pipecolic acid, 3,4,5-trimethoxyphenyl-β-D-glucopyranoside, malic acid, and fumaric acid were disappeared at the 7d of *S. cerevisiae* fermentation. Venn diagram analysis demonstrated that there were 76 common differential metabolites among different comparison groups (Fig. 3D).

3.4. The correlations between metabolites and BC yield

The large differences between groups imply that the level of BC yield was closely associated with the changes in metabolic profile in F1 and F7. To identify DMs that may be involved in the metabolism and BC synthesis of *K. nataicola* Y19, the Spearman correlation coefficient was used to screen the DMs that are significantly correlated with BC yield. With a threshold of Spearman correlation coefficient >0.85, $p < 0.05$, or <-0.85, $p < 0.05$, 38 significant differential metabolites were screened out. Among these metabolites, 17 metabolites showed a positive correlation and belonged to alkaloids, amino acids and derivatives, organic acids, and phenolic acids. Additionally, 21 metabolites showed a negative correlation and belonged to categories including alkaloids, amino

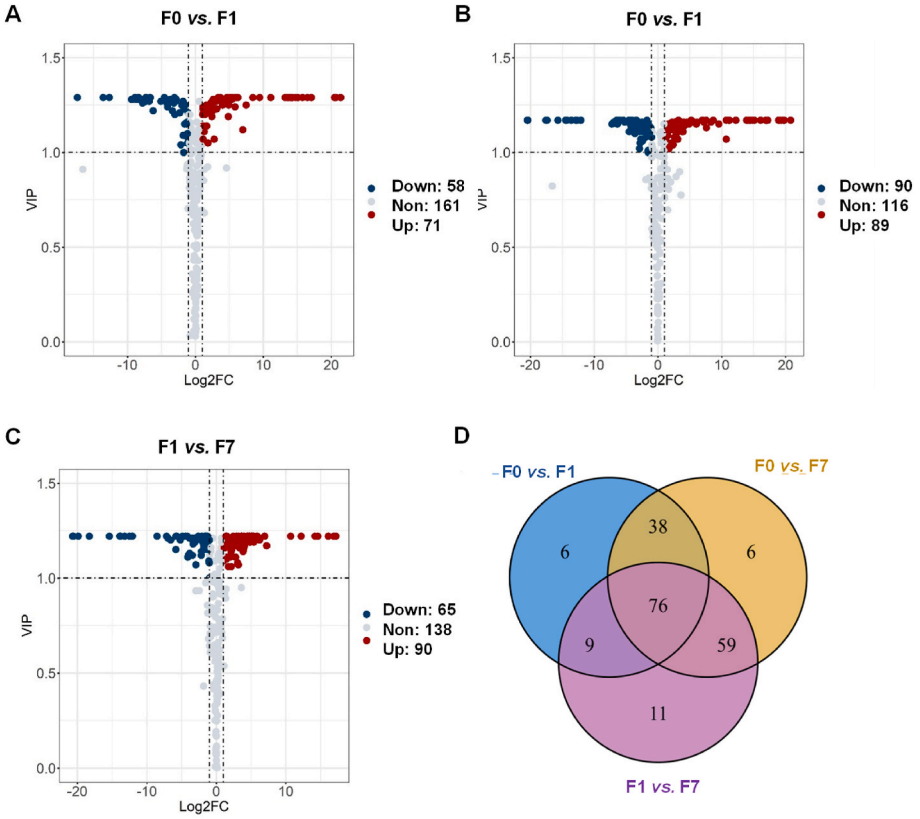

Fig. 3. Distribution of metabolites in unfermented coconut water (F0) and coconut water fermented by *S. cerevisiae* 84-3 for 1d (F1) and 7d (F7). Volcano plot representing the relationship between fold change and VIP in F0 *vs.* F1 (A), F0 *vs.* F7 (B), F7 *vs.* F1 (C). (D) Venn diagram illustrating the overlapping and specific differential metabolites for three comparison groups.

Table 1
Significantly regulated metabolites between unfermented coconut water and coconut water fermented by *S. cerevisiae* 84-3 for 1d.

Formula	Compounds	Class	VIP	Log2FC	Type	*p*-value
$C_6H_{12}O_4$	(Rs)-mevalonic acid	Organic acids	1	21.42	up	0.00
$C_4H_4O_4$	Fumaric acid	Organic acids	1	20.7	up	0.01
$C_9H_8O_3$	Phenylpyruvic acid	Organic acids	1	20.51	up	0.00
$C_5H_{12}O_5$	D-arabitol	Others	1	17.13	up	0.02
$C_9H_7NO_2$	Indole-2-carboxylic acid	Alkaloids	1	16.48	up	0.00
$C_4H_8O_3$	2-hydroxybutanoic acid	Organic acids	1	15.81	up	0.01
C_8H15NO_3	Hexanoyl glycine	Amino acids and derivatives	1	15.15	up	0.11
$C1_3H1_4N_2O_3$	Acetyltryptophan	Amino acids and derivatives	1	14.76	up	0.00
$C_{10}H1_3N_5O_3$	Deoxyadenosine	Nucleotides and derivatives	1	14.35	up	0.00
$C_7H_{12}N_2O_4$	N-α-acetyl-L-glutamine	Amino acids and derivatives	1	14.2	up	0.03
$C_9H_{10}O_3$	Phenyllactate	Organic acids	1	13.98	up	0.00
$C_9H_{10}O_3$	3-(4-hydroxyphenyl)-propionic acid	Phenolic acids	1	13.81	up	0.00
$C_9H_{10}O_3$	L-(−)-3-phenyllactic acid	Organic acids	1	13.78	up	0.00
$C_{12}H_{24}O_3$	12-hydroxydodecanoic acid	Lipids	1	13.45	up	0.00
$C_{10}H1_3N_5O_6$	8-hydroxyguanosine	Nucleotides and derivatives	1	13.16	up	0.00
$C_5H_{10}O_3$	2-hydroxy-2-methylbutyric acid	Organic acids	1	11.12	up	0.00
$C1_6H_{32}O_4$	10,16-dihydroxy-palmitic acid	Lipids	1	9.46	up	0.03
$C_6H1_3NO_2$	L-isoleucine	Amino acids and derivatives	1	−8.38	down	0.00
$C_6H1_4N_2O_2$	L-(+)-lysine	Amino acids and derivatives	1	−8.44	down	0.00
$C_6H1_3NO_2$	L-leucine	Amino acids and derivatives	1	−8.64	down	0.00
$C_6H1_3NO_2$	α-aminocaproic acid	Amino acids and derivatives	1	−8.77	down	0.00
$C_6H_9NO_5$	N-acetylaspartate	Amino acids and derivatives	1	−8.92	down	0.00
$C_{11}H_{12}N_2O_2$	Tryptophan	Amino acids and derivatives	1	−9.29	down	0.00
$C_5H_{11}NO_2S$	L-methionine	Amino acids and derivatives	1	−9.42	down	0.00
$C_8H1_4N_2O_5$	(5-L-glutamyl)-L-amino acid	Amino acids and derivatives	1	−12.72	down	0.03
$C_3H_7NO_3$	Serine	Amino acids and derivatives	1	−13.62	down	0.00
$C_6H_7N_5$	1-methyladenine	Nucleotides and derivatives	1	−17.43	down	0.00

Table 2
Significantly regulated metabolites between coconut water fermented by *S. cerevisiae* 84-3 for 1d and 7d.

Formula	Compounds	Class	VIP	Log2FC	Type	*p*-value
$C_4H_8O_3$	α-hydroxyisobutyric acid	Organic acids	1.22	17	up	0.00
$C_10H_{12}N_4O_5$	9-(β-D-Arabinofuranosyl)hypoxanthine	Nucleotides and derivatives	1.22	17	up	0.00
$C_4H_8O_3$	(S)-2-hydroxybutanoicacid	Organic acids	1.22	17	up	0.00
$C_4H_6O_6$	L-(+)-tartaric acid	Organic acids	1.22	16	up	0.00
$C_8H_{14}N_2O_5$	(5-L-glutamyl)-L-amino acid	Amino acids and derivatives	1.22	15	up	0.00
$C_6H_9NO_5$	N-acetylaspartate	Amino acids and derivatives	1.22	14	up	0.00
$C_9H_8O_2$	Cinnamic acid	Phenolic acids	1.22	12	up	0.00
$C_10H_{18}O_4$	Sebacate	Organic acids	1.22	11	up	0.00
$C_5H_{13}NO$	Choline	Alkaloids	1.17	7	up	0.00
$C_4H_5N_3O$	Cytosine	Nucleotides and derivatives	1.22	7	up	0.00
$C_6H_8O_7$	Citric acid	Organic acids	1.22	−9	down	0.02
$C_{32}H_{42}O_{16}$	Pinoresinol diglucoside	Lignans	1.22	−12	down	0.00
$C_{17}H_{24}O_9$	Syringin	Phenolic acids	1.22	−13	down	0.00
$C_10H_{12}N_5O_6P$	Cyclic AMP	Nucleotides and derivatives	1.22	−13	down	0.00
$C_{26}H_{32}O_{11}$	Pinoresinol-hexose	Lignans	1.22	−14	down	0.01
$C_{26}H_{32}O_{11}$	Terpineol monoglucoside	Lignans	1.22	−14	down	0.00
$C_{13}H_{16}O_9$	2,5-Dihydroxy benzoic acid O-hexside	Phenolic acids	1.22	−15	down	0.00
$C_6H_{11}NO_2$	Pipecolic acid	Amino acids and derivatives	1.22	−18	down	0.01
$C_{15}H_{22}O_8$	3,4,5-trimethoxyphenyl-β-D-glucopyranoside	Phenolic acids	1.22	−20	down	0.00
$C_4H_6O_5$	Malic acid	Organic acids	1.22	−21	down	0.00
$C_4H_4O_4$	Fumaric acid	Organic acids	1.22	−21	down	0.01

acids, and derivatives, free fatty acids, nucleotides and derivatives, organic acids, and phenolic acids (Fig. 4).

Among the 17 significantly positively correlated metabolites, indole-2-carboxylic acid, alanyl leucine, hexanoyl glycine, phenylpyruvic acid, mevalonic acid, and fumaric acid were produced after *S. cerevisiae* 84-3 fermentation. Among them, the relative content levels of phenylpyruvic acid and (Rs)-mevalonic acid, which belongs to organic acids, were very high in F1 which was more than 50 times than that of F0 and F7. Interestingly, fumaric acid only formed in F1. Compared with the content of the aforementioned organic acids, the relative content of amino acids such as alanylleucine, hexanoyl glycine, L-phe, and L-tyramin in F1 and F7 were much lower, whose content was only 0.23%–6% of organic acids. Specifically, the content of dihydroxybenzoic acid and gentisic acid increased by 17.21% and 21.13% respectively on the first day of *S. cerevisiae* 84-3 fermentation. However, with the extension of the fermentation time to 7d, they were largely consumed, and the final relative content was only 58.16% and 58.40% of the initial content, respectively. Other metabolites such as indole-3-carboxaldehyde, tryptophol, vanillic acid, and gentisic acid had higher relative content levels in F1 than the other two groups.

Out of the 21 significantly negatively correlated metabolites, more than half of them belong to the category of amino acids and their derivatives. Those negatively metabolites were divided into three categories: (1) content of metabolite was high in F0 and absolutely metabolized by *S. cerevisiae* 84-3 fermentation. These kinds of metabolites including serine and 1-methyladenine; (2) content of metabolites was extremely higher that other metabolites in F0 and substantial reduced for more than 100 times but still existed in F1 and F7 at low level. Those kinds of metabolites including L-histidine, L-glutamine, L-proline, L-glutamine, L-(+)-lysine. (3) Content of metabolites was high

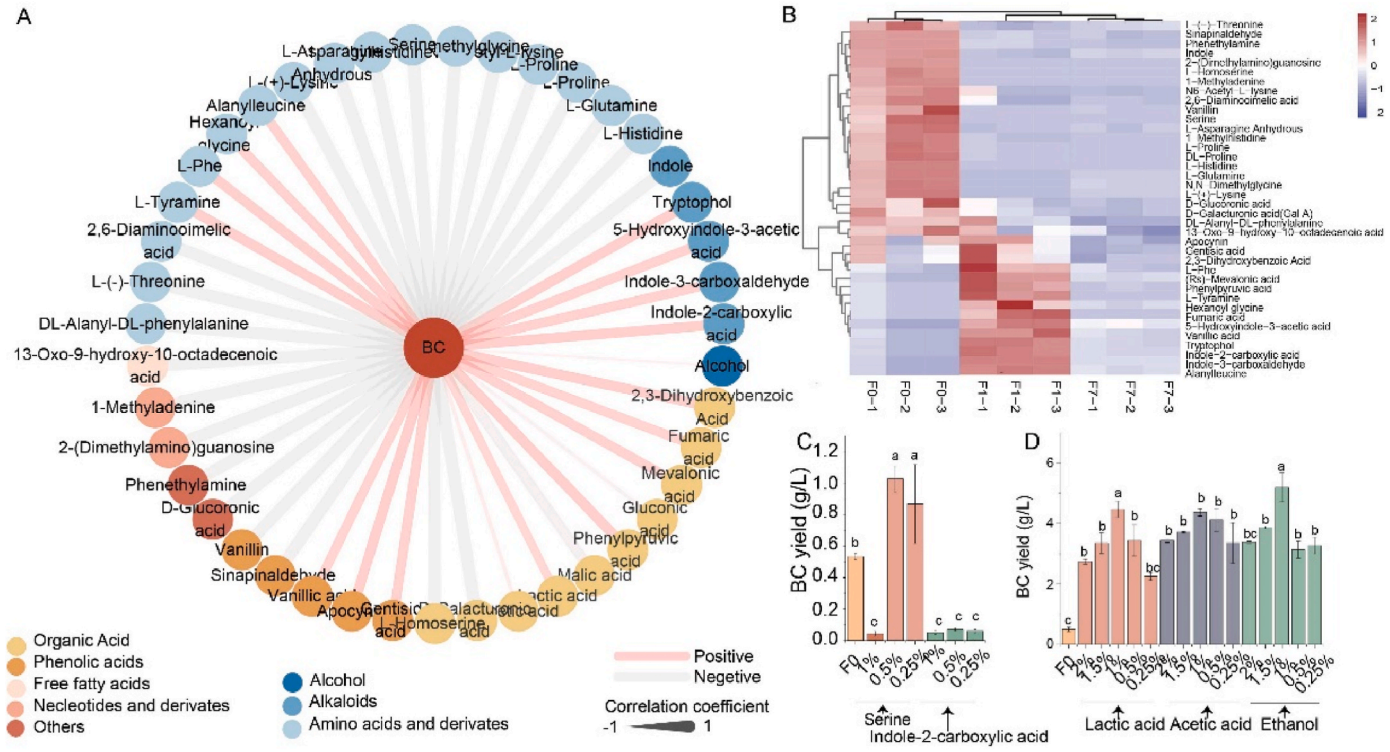

Fig. 4. BCE yield-related metabolites and their preliminary validation. (A) Correlation between metabolites between BC yield. Edge thickness corresponds to a correlation between the nodes based on Spearman's rank correlation coefficient. (B) Relative abundance of high correlative metabolites. Red/blue color represents the increase/decrease in content. (C) and (D) BC yield of *K. nataicola* Y19 in coconut water supplemented with different concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in F0 and decreased for 1.5–45 times after *S. cerevisiae* 84-3 fermentation. Those kinds of metabolites including indole, 2,6-diaminooimelic acid, L-(−)-threonine, DL-alanyl-DL-phenylalanine, N6-acetyl-L-lysine, L-homoserine, D-galacturonic acid, vanillin, and D-glucoronic acid. The correlation between lactic acid, acetic acid, ethanol and BC yield is also calculated and the results showed a significant positive correlation between lactic acid content and BC yield, and a positive correlation but not significant between acetic acid content and BC yield. However, the correlation between the ethanol and BC yield during pre-fermentation was not observed.

3.5. Verification experiments results

Here, three types of metabolites with positive correlation (2-indolecarboxylic acid, acetic acid, lactic acid), negative correlation (serine), and no correlation but widely added in medium to produce BC yield (ethanol) were chosen to further verify their effects on BC yield (Fig. 4C and D). The results showed that the low concentration of serine (0.5%–0.25%) could improve BC yield by 1.92 times and 1.62 times but higher concentration of serine (1%) significantly suppressed the BC production. On the other hand, 2-indolecarboxylic acid at concentration of 1%, 0.5% 0.25% was suppress the BC production which is contrast to the correlation analysis result. In addition, 1% lactic acid, 1% acetic acid and 1% ethanol significantly increased the BC yield of *K. nataicola* Y19 to 4.47 ± 0.27 g/L, 5.2 ± 0.48 g/L and 4.37 ± 0.12 g/L, respectively, which were more than 6 time higher than BC yield in F0. However, the BC yield of *K. nataicola* Y19 was decreased when the concentrations were higher than 1%. Above all, the BC yield was closely related to the type of metabolites and controlled by their concentration.

4. Discussion

In this study, coconut water fermented by *S. cerevisiae* 84-3 significantly enhanced the BC yield and the BC yield is closely related to the fermentation time. Here, acetic acid and lactic acid had a positive correlation with BC yield. Previous studies have shown that lactic acid and acetic acid could enhance BC production (Lin et al., 2020). Specifically, previous research examined that acetic acid at a concentration of 20 g/L and lactic acid at 0.15% (v/v) increased the BC yield of *Acetobacter xylinum* by 4 and 4.5 times, respectively (Bae et al., 2004). Lactic acid promoted bacterial proliferation and growth in the early stages of fermentation (Bae et al., 2004). In this study, ethanol was formed during the *S. cerevisiae* 84-3 fermentation and might indirectly take part in BC synthesis because it can be utilized as energy sources through the TCA cycle, facilitating ATP secretion and providing additional energy for BC-producing bacteria (Cielecka et al., 2021). Additionally, ethanol can enhance the activity of glucose acid kinase and fructokinase, while reducing the activity of glucose kinase and glucose-6-phosphate dehydrogenase. Those processes promote the conversion of glucose into BC precursor substances (Cielecka et al., 2021; Yunoki et al., 2007). However, promoting effects of metabolites were limited by their concentrations as the enhancing effects gradually diminished as concentrations increased, which aligned with previous studies (Cielecka et al., 2021; Molina-Ramírez et al., 2018).

Further analysis revealed significant correlations between multiple classes of metabolites, such as alkaloids, amino acids and derivatives, organic acids, nucleotides and derivatives, and phenolic acids, with the BC metabolism and synthesis of *K. nataicola* Y19. Organic acids are various and play a role in regulating the growth of various microorganisms and the secretion of target products (Hsiao and Siebert, 1999; Lyu et al., 2019). For example, phenylpyruvic acid, which showed significant changes in this study, acts as a precursor to phenyllactic acid. Phenyllactic acid has various biological functions, including interfering with the formation of *Lactobacillus plantarum*'s lipids (Ilavenil et al., 2015), and participating in quorum sensing and biofilm formation (Chatterjee et al., 2017). Fumaric acid, another metabolite that

significantly changed during pre-fermentation, is an important component in the TCA cycle. The presence of fumaric acid along with these metabolites indicates that F1 may be involved in the energy metabolism associated with BC synthesis in *K. nataicola* Y19. Mevalonic acid is existed in animals, plants, and microorganisms and is involved in various metabolic pathways in eukaryotes. It is an important precursor for the synthesis of steroids and terpenoids, such as isoprene, carotenoids, and artemisinin (Farjaminezhad and Garoosi, 2020; Matsumoto et al., 2017; Miao et al., 2020). Terpenoids play crucial roles in maintaining membrane fluidity (Wriessnegger and Pichler, 2013). On the other hand, BC production is accomplished with the secretion, assembly, and crystallization of glucose outside bacterial cells. Cellulose synthesized by *K. nataicola* Y19 is extruded outward through micro-pores in the cell membrane. A high level of mevalonic acid may promote the formation of terpenoids and be correlated with the cell fluidity of *K. nataicola* Y19 and the efficient expulsion of BC.

Amino acids and their derivatives serve as crucial precursors in various metabolic pathways and play significant roles in microbial metabolism and function (Nimbalkar et al., 2019). In a study conducted by Gomes et al. (2020), it was found that aspartic acid, phenylalanine, and serine enhanced BC production in a static culture environment. Son et al. (2003) found that the addition of tyrosine, valine, methionine, isoleucine, and glycine significantly inhibited BC production in *Acetobacter* sp. In our recently published study (Lin et al., 2022), we demonstrated that the addition of serine significantly reduces BC production in natural fermented coconut water, whereas the addition of aspartic acid, glutamic acid, methionine, and isoleucine promotes BC production. These findings suggest that amino acids have a significant impact on BC production, but the effects may differ across bacterial strains. Meanwhile, high concentrations of amino acids can be toxic to microorganisms (Rawson, 1985), thus the types and concentrations of amino acids could potentially interfere BC production. In this study, the content of histidine, glutamine, L-proline, DL-proline, and L-lysine was high in F0 but significantly decreased after pre-fermentation with 84-3, while serine was completely consumed and metabolized at 7d of *S. cerevisiae* 84-3 fermentation. Those results imply that high concentrations of these particular amino acids may have inhibitory effects on the growth, metabolism, and synthesis of BC in *K. nataicola* Y19. On the other hand, the appropriate concentration of these particular amino acids may participate in and regulate BC synthesis metabolism in *K. nataicola* Y19. The exogenous addition experiments in this study also gave powerful evidence to support those views. In addition, some metabolites might play negative roles in physiological function of microorganism. For example, N, N-dimethylglycine, as a derivative of amino acids, significantly decreased after pre-fermentation with *S. cerevisiae* 84-3, can disrupt the integrity of cell membranes (Zou et al., 2016), which suggested that it may interfere with the secretion and expulsion of BC in *K. nataicola* Y19 by altering the structure and function of the cell membrane.

5. Conclusion

In sum, coconut water fermented by *S. cerevisiae* 84-3 for 1d had the greatest promoting effect on BC yield. With the increase of fermentation time of *S. cerevisiae* 84-3, the promoting effects of fermented coconut water were attenuated which is significantly related to the constitution of metabolites and their concentration. Those metabolites play different roles in energy sources, potential signaling molecules and precursor and participate in energy metabolism, BC synthesis, protein synthesis and metabolism and integrity of cell membranes. This study mainly focused on the composition of fermented coconut water and relevant experiments can be designed to explore the underlying mechanism regulation of *K. nataicola* in coconut water fermented by *S. cerevisiae* 84-3.

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CRediT authorship contribution statement

Shuangwen Fei: Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Meijuan Fu:** Conceptualization, Methodology, Investigation, Visualization. **Jiamu Kang:** Writing – review & editing. **Jiaxi Luo:** Methodology, Investigation. **Yanmei Wang:** Investigation. **Jia Jia:** Investigation. **Sixin Liu:** Conceptualization, Resources, Formal analysis, Funding acquisition, Writing – review & editing. **Congfa Li:** Funding acquisition, Supervision, Project administration.

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.

Abbreviations

BC	bacterial cellulose
F0	unfermented coconut water
F1	coconut water fermented by *Saccharomyces cerevisiae* 84-3 for 1d
F7	coconut water fermented by *Saccharomyces cerevisiae* 84-3 for 7d
UPLC-ESI-MS/MS	Ultra-performance liquid chromatography combined with mass spectrometry
DMs	significantly different metabolites
QC	quality control
PCA	principal component analysis
VIP	value of variable importance
FC	fold change
OPLS-DA	orthogonal signal correction and partial least squares-discriminant

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100761.

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