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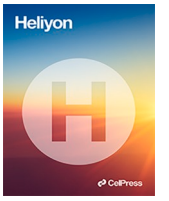

Research article

Utilizing deep ocean water in yeast fermentation for enhanced mineral-rich biomass production and fermentative regulation by proteomics modulation

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ABSTRACT

Deep Ocean Water (DOW) is rich in minerals and serves as a natural source of nutrients. However, due to the inorganic nature of these minerals, cultivating yeast in DOW could aid in the fermentation process, and simultaneously, the yeast can assimilate the minerals from DOW, resulting in a mineral-enriched yeast biomass. Focusing on three DOW sources off the eastern coast of Taiwan (TT-1, HL-1, HL-2), we fermented various yeast strains of *Saccharomyces cerevisiae*. Therefore, this study investigates the effects of DOW on yeast growth, alcohol dehydrogenase activity, and the biological absorption of mineral ions by the yeast. Additionally, this research employs two-dimensional electrophoresis techniques to examine how the absorbed minerals influence the regulation of yeast proteins, thereby affecting biomass and metabolism. In the result, *S. cerevisiae* BCRC 21689 demonstrated a remarkable ability to bio-absorb minerals such as magnesium, calcium, potassium, and zinc from DOW, enhancing its growth and fermentation performance. Proteomic analysis revealed significant shifts in the expression of 21 proteins related to glycolytic and energy metabolism, alcohol metabolism, and growth regulation, all influenced by DOW's mineral-rich environment. This indicates that DOW's mineral content is a key factor in upregulating essential enzymes in glycolytic metabolism and alcohol dehydrogenase. An increase in proteins involved in synthesis and folding processes was also observed, leading to a substantial increase in yeast biomass. This study underscores the potential of DOW as a natural enhancer in yeast fermentation processes, enriching the yeast with diverse minerals and modulating proteomic expression to optimize yeast growth and fermentation.

1. Introduction

Deep ocean water (DOW), known for its nutrient-rich properties, has been highlighted in numerous studies for its various health benefits. These include improving dermatitis [1], combating arteriosclerosis [2], cardiovascular diseases [3,4], and reducing obesity [5]. Beyond these health effects, the application of DOW in the production of health-promoting microbial fermentation can be

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beneficial for the development of multifunctional health foods. By leveraging DOW, fermentation efficiency can be increased, production costs can be reduced, and both the value and health functions of products can be significantly enhanced [6]. Previous studies by our research team have established the use of DOW in the production of functional microorganisms such as red yeast rice, Antrodia camphorata, Cordyceps militaris, and Epichloë festucae, creating a comprehensive database. In studies on red yeast rice, DOW culture resulted in higher efficacious component content of monascin and ankaflavin. Minerals in DOW such as magnesium, zinc, and iron were found to increase the content of these components while also reducing the levels of the nephrotoxic and hepatotoxic compound citrinin [7–9]. Our team has also applied DOW to enhance the fermentation production of *Antrodia cinnamomea* mycelium, improving the hepatoprotective effects and mitigating Alzheimer's disease [10]. Furthermore, in the application of DOW in *Cordyceps militaris* fermentation, mineral nutrients promoted mycelial growth and cordycepin production [11,12], thereby improving the effects on hepatic fibrosis progression. In the cultivation of *Epichloë festucae*, the minerals from DOW were absorbed by the organisms and converted into organic minerals. Specifically, organic magnesium, which can be effectively absorbed to supplement brain magnesium levels, promoted the expression of magnesium transport proteins in the brain, allowing for increased magnesium ion entry into neurons. Supplementing with magnesium reduced the expression of factors associated with Alzheimer's disease, ultimately improving memory and learning capabilities [13,14].

DOW has become a valuable resource in the food fermentation industry. It has been traditionally used in various fermentation processes such as winemaking and bread fermentation, often by adding DOW to yeast fermentation to enhance flavor and efficiency. However, the source and concentration of DOW can affect the fermentation results, and different yeast strains may have different adaptabilities and needs regarding DOW. To date, there has been no related research on the application of DOW in yeast fermentation. Therefore, exploring the effects of DOW on yeast growth, alcohol dehydrogenase activity, and proteomic expression during fermentation is crucial for the future development of the food fermentation industry. This study primarily investigates the application of DOW in yeast fermentation production and protein expression. Rich DOW resources off the eastern coast of Taiwan, specifically the Pacific Ocean, will be utilized. Experiments will be conducted using DOW from Hualien, sourced from 600 m depth, and from Taitung, sourced from 300 m depth. By fermenting three types of yeast with different sources of DOW, we aim to compare not only yeast growth and alcohol dehydrogenase activity but also to study whether the yeast can absorb mineral nutrients from the DOW. This research aspires to understand the mechanisms by which DOW affects yeast physiological activity, providing valuable guidance for optimizing the food fermentation process using DOW.

2. Materials and methods

2.1. Chemicals

Yeast mold (YM) agar and broth were purchased from Difco Laboratories (Detroit, MI, USA). Magnesium chloride was purchased from Showa Chemical Industry (Tokyo, Japan). Ethanol (95 %) was purchased from the Taiwan Tobacco and Liquor Corp. (Taipei, Taiwan). All other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Source and preparation of DOW

In this study, the concentrated DOW used was sourced from Taitung and Hualien in Taiwan. The DOW from below 300 m in the Taitung area was provided by the Eastern DOW Innovation Research and Development Center, named as TT-1 concentrate. The DOW from the Hualien area, sourced from below 600 m, was purchased Taiwan Yes Ocean Deep Water Co., Ltd. (Hualien, Taiwan, ROC) and Kuang-Long Ocean Biotech Co., Ltd., (Hualien, Taiwan, ROC) named as HL-1 and HL-2 concentrates, respectively. The mineral composition of each concentrate is shown as follow: In terms of magnesium (Mg) concentration, HL-1 was the highest at 100,179 mg/L, followed by TT-1 at 84,758 mg/L, and then HL-2 at 80,865 mg/L. For calcium (Ca) concentration, TT-1 was significantly higher than the other two, reaching 37,228 mg/L, whereas the calcium concentrations in HL-1 and HL-2 were only 20 mg/L and 38 mg/L, respectively. The concentrations of potassium (K) and sodium (Na) were highest in the HL-2 concentrate, at 23,956 mg/L and 26,370 mg/L, respectively. Other minerals like zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), and molybdenum (Mo), although not varying greatly, also differed among the three concentrates. These differences might be due to the seawater sources or different processing methods.

Due to the high concentration of minerals in the DOW concentrates, to make them suitable for yeast cultivation, this study diluted the three types of DOW concentrates to magnesium ion concentrations of 200 mg/L, 400 mg/L, 600 mg/L, and 800 mg/L. The effects of different sources and concentrations of DOW on yeast growth, enzyme activity, and proteomics expression were explored.

2.3. Yeast sources

The yeast strains used in this experiment were obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan). The strains included *S. cerevisiae* BCRC 23144, *S. cerevisiae* BCRC 21833, and *S. cerevisiae* BCRC 21685. They were cultured in YPDA medium at 25 °C for one week.

2.4. Liquid cultivation

After activation of the strains, liquid cultures were conducted using DOW mineral solutions. The impact of DOW mineral solutions

of different origins and concentrations on yeast cell growth and composition changes was investigated. Three sources of DOW were used to culture the yeast, each yeast strain was cultured in a maltodextrin solution culture medium (containing 10 % maltodextrin and 1 % yeast extract) with 10, 20, 30, and 40 times dilutions of DOW minerals. After 48 h of colony growth on a flat culture, the cultures were incubated at 30 °C and 120 rpm for 72 h. The cell suspension was subsequently centrifuged, and the resulting precipitate was dried for 24 h in an empty culture dish, with the dry weight of the biomass recorded.

2.5. Examination of SOD-like activity in yeast and mineral absorption

SOD-like activity in the fermentation fluid was analyzed using a commercial kit (SD125, RANDOX, Ulster, UK). Alcohol dehydrogenase of the fermentation liquid of yeast was analyzed using the alcohol dehydrogenase activity assay kit (E-BC-K763-M, Elabscience Biotechnology Co., Ltd., Wuhan, China). The analysis method was performed according to the instructions of the kit.

2.6. Mineral analysis

Major elements (Ca, Mg, K, P) concentrations were determined by ICP-OES. Trace elements (Zn, Fe, Mn, Cu, Mo) concentrations were measured by quadrupole-ICP-MS (Agilent 8900) in He collision mode. The ICP-MS is calibrated using NIST multi-element standards containing the analytes of interest. A series of six calibration standards was conducted at concentrations 0, 0.01, 0.1, 1, 5, 10 mg/L.

Samples were dried at 80 °C in an oven until constant weight and then ground into fine powder. About 0.2 g of sample powder was dissolved in 10 mL of nitric acid, heated to boiling, cooled and transferred to a 50 mL volumetric flask as sample solution. Major elements (Ca, Mg, K, P) concentrations were determined by ICP-OES (PerkinElmer Optima 8300, Shelton, Connecticut, USA). Appropriate emission wavelengths were selected, such as Ca 317.933 nm, Mg 279.553 nm, K 766.491 nm, P 178.221 nm. Yb was used as an internal standard to correct for matrix effects and ionization interferences. NIST multi-element standard (SRM 1640a, Gaithersburg, Maryland, USA) was used for calibration, with concentration range of 0.1–10 mg/L. Trace elements (Zn, Fe, Mn, Cu, Mo) concentrations were measured by quadrupole- ICP-MS (Agilent 8900, Santa Clara, California, USA) in He collision mode. Suitable mass numbers were chosen to avoid isotopic interferences, such as Zn 67, Fe 56, Mn 55, Cu 63, Mo 95. HPS ICP-MS multi-element solution standard (ICP-MS-68A, Charleston, South Carolina, USA) was used for calibration, with concentration range of 0.01–1 μg/L. This standard was traceable to NIST SRM 3100 series. Blank solution and quality control samples were used to monitor the stability and accuracy of calibration.

2.7. Extraction and quantification of yeast cell proteins

After centrifugation of the fermentation liquid, the supernatant was discarded, and the cell sediment was weighed (Balance XS105, Mettler-Toledo, MA, USA) and extracted for cellular protein using 2DE lysis buffer (8 M Urea, 4 % CHAPS). Protein quantification was performed using the BCA protein assay (Thermo Fisher Scientific, MA, USA).

2.8. Two-dimensional electrophoresis analysis and resolution

110 μg of protein samples were loaded on the Ettan IPGphor 3 IEF system (GE Healthcare Life Science), and first-dimension electrophoresis was performed using 13 cm Immobiline DryStrip pH 3–10 (maximum voltage: 6700 v). The strips were then mixed with 65 mM dithiothreitol and 135 mM iodoacetamide and transferred onto a vertical gel (12 % SDS-PAGE, Hoefer SE600; GE Healthcare Life Science) for second-dimension separation, and finally visualized with Silver staining. Targeted gel spots were excised, destained, reduced at 60 °C with 10 mM dithiothreitol for 45 min, then cysteine-blocked at 25 °C with 55 mM iodoacetamide for 30 min. Samples were digested with trypsin at 37 °C for 16 h, and the resulting peptides were vacuum-dried for later use.

2.9. Identification of protein expression

Gel spots for targeted analysis were excised and destained. They were then reduced at 60 °C with 10 mM dithiothreitol for 45 min and cysteine-blocked at 25 °C with 55 mM iodoacetamide for 30 min. The samples were digested with trypsin at 37 °C for 16 h, and the resulting peptides were vacuum-dried for later use.

The digested peptide samples were diluted with HPLC buffer A (0.1 % formic acid) and loaded onto a reverse-phase column (Zorbax 300SB-C18, 0.3 × 5 mm; Agilent Technologies). The peptides were then desalted on the column (Waters BEH 1.7 μm, 100 μm I.D. × 10 cm, 15 μm tip) using a multistage gradient of HPLC buffer B (99.9 % acetonitrile/0.1 % formic acid) at a flow rate of 0.3 μl/min for approximately 70 min. The LC system was coupled to a linear ion trap mass spectrometer (Orbitrap Elite ETD; Thermo Fisher), operated using X calibur 2.2 software (Thermo Fisher). Full-scan MS was performed in the Orbitrap, ranging from 400 to 2000 Da, with a resolution of 120,000 at m/z 400. Internal calibration was performed using the ion signal of protonated dodecamethylcyclohexasiloxane ions at *m/z* 536.165365. A single MS scan was performed for the 20 most abundant parent ions in the preview MS scan following 20 data-dependent MS/MS scanning events. Selected *m/z* values for MS/MS were dynamically excluded for 40 s with a relative mass window of 15 ppm. Electrospray voltage was set to 2.0 kV, and the capillary temperature was set to 200 °C. Auto gain control for MS and MS/MS was set to 1000 ms (full scan) and 200 ms (MS/MS), or 3 × 106 ions (full scan) and 3000 ions (MS/MS), for maximum cumulative time or ion count.

Electrophoretic gels were photographed and scanned for analysis using Epson Perfection V800 Photo software (Epson, Epson Perfection V800 Photo., Indonesia). Protein spots showing differential expression between groups were analyzed using Proteome Discoverer software (Thermo Fisher) and searched against the Uniprot database using the Mascot search engine (Matrix Science). For peptide identification, a complete peptide mass tolerance of 10 ppm and a CID fragment ion tolerance of 0.5 Da were allowed, with two missed cleavages allowed for trypsin digestion: oxidation of methionine and acetylation (protein N-terminus) as variable modifications; carbamidomethylation (cysteine) as a static modification. PSMs (Peptide Spectrum Matches) were filtered for high confidence, with Mascot search engine rankings at first place for peptide identifications, ensuring an overall false discovery rate below 0.01. Proteins with single peptide hits were excluded.

3. Results

3.1. The effect of DOW on the growth and alcohol dehydrogenase activity in various yeast strains

S. cerevisiae BCRC 23144, a wine fermentation yeast strain isolated for oenological purposes, was assessed for potential applications in growth and metabolite production enhancement using different sources of DOW (Table 1). Post-fermentation, compared to the ultrapure water group (UPW), all concentrations of TT-1 DOW failed to enhance biomass growth ($p < 0.05$). However, TT-1 at 10X concentration significantly increased alcohol dehydrogenase activity ($p < 0.05$); at all concentrations of HL-1, there was no increase in biomass, but DOW-HL-1 at 20X, 30X, and 40X concentrations significantly enhanced alcohol dehydrogenase activity ($p < 0.05$). Conversely, none of the concentration multiples of DOW-HL-2 effectively improved biomass or alcohol dehydrogenase activity ($p < 0.05$). In summary, TT-1, HL-1, and HL-2 did not enhance the growth of *S. cerevisiae* BCRC 23144 in fermentation production applications, but TT-1 at 10X and HL-1 at 10X, 20X, 30X, and 40X effectively increased the strain's alcohol dehydrogenase activity ($p < 0.05$).

S. cerevisiae BCRC 21833, a yeast strain suitable for beer fermentation, was also evaluated for its growth and metabolite production using various sources of DOW (Table 2). Only TT-1 at 10X concentration was effective in increasing biomass ($p < 0.05$), whereas the addition of TT-1 significantly reduced alcohol dehydrogenase activity ($p < 0.05$), suggesting it may be less suitable for alcohol fermentation in this strain; all concentrations of HL-1 were ineffective in enhancing both biomass and alcohol dehydrogenase activity; similarly, HL-2 at all concentrations failed to increase both biomass and enzyme activity. Collectively, for *S. cerevisiae* BCRC 21833 fermentation, only TT-1 at 10X concentration improved biomass growth, with no DOW treatments effectively increasing alcohol dehydrogenase activity. These results indicate that the three different sources of DOW are not suitable for fermentation efficacy in the *S. cerevisiae* BCRC 21833.

S. cerevisiae BCRC 21685, a yeast strain used for yellow rice wine production, was further evaluated for its growth and metabolite production potential with different DOW sources (Table 3). Compared to UPW, TT-1 at 10X, 30X, and 40X concentrations effectively increased biomass ($p < 0.05$), but had no significant effect on enhancing alcohol dehydrogenase activity ($p < 0.05$); all concentrations of HL-1 effectively increased biomass ($p < 0.05$), but had no significant impact on alcohol dehydrogenase activity. In the fermentation assessment with HL-2, all concentrations failed to increase both biomass and enzyme activity. Therefore, for *S. cerevisiae* BCRC 21685 fermentation production with TT-1 and HL-1, while TT-1 at 10X, 30X, and 40X and all concentrations of HL-1 effectively increased biomass, none of the DOW media enhanced alcohol dehydrogenase activity.

3.2. Microbial absorption and utilization of mineral elements in DOW

The minerals and trace elements contained in DOW play a crucial role in the growth and metabolic processes of a variety of microorganisms. To investigate their absorption and utilization by different yeast strains, we analyzed the changes in mineral content in

Table 1
The effect of DOW on the biomass and alcohol dehydrogenase activity of *S. cerevisiae* BCRC 23144 under subnerged fermentation.

Cultivation water	Biomass (g/100 mL)	Alcohol dehydrogenase activity (U/L)
UPW	0.287 ± 0.029^{c}	2.795 ± 0.841^{a}
TT-1 10X	0.200 ± 0.020^{a}	4.589 ± 0.892^{b}
TT-1 20X	0.233 ± 0.012^{b}	3.017 ± 0.209^{a}
TT-1 30X	0.240 ± 0.000^{b}	3.182 ± 0.518^{ab}
TT-1 40X	0.253 ± 0.012^{b}	3.503 ± 0.326^{ab}
HL-1 10X	0.200 ± 0.016^{c}	6.688 ± 1.598^{bc}
HL-1 20X	0.230 ± 0.009^{a}	8.400 ± 0.437^{c}
HL-1 30X	0.240 ± 0.000^{ab}	7.799 ± 0.858^{bc}
HL-1 40X	0.250 ± 0.009^{bc}	6.112 ± 1.482^{b}
HL-2 10X	0.267 ± 0.012^{a}	2.556 ± 0.038^{a}
HL-2 20X	0.240 ± 0.035^{a}	2.960 ± 0.136^{a}
HL-2 30X	0.247 ± 0.012^{a}	3.166 ± 0.138^{a}
HL-2 40X	0.273 ± 0.031^{a}	2.795 ± 0.209^{a}

Table 2
The effect of DOW on the biomass and alcohol dehydrogenase activityof *S. cerevisiae* BCRC 21833 under subnerged fermentation.

Cultivation water	Biomass (g/100 mL)	Alcohol dehydrogenase activity (U/L)
UPW	0.203 ± 0.021^{ab}	4.647 ± 0.776^{b}
TT-1 10X	0.240 ± 0.026^{a}	2.688 ± 0.341^{a}
TT-1 20X	0.257 ± 0.006^{c}	2.795 ± 0.304^{a}
TT-1 30X	0.250 ± 0.020^{bc}	3.379 ± 0.528^{a}
TT-1 40X	0.220 ± 0.030^{a}	2.622 ± 0.430^{a}
HL-1 10X	0.253 ± 0.006^{b}	4.852 ± 0.898^{a}
HL-1 20X	0.247 ± 0.023^{b}	4.490 ± 0.827^{a}
HL-1 30X	0.200 ± 0.017^{ab}	5.108 ± 0.528^{a}
HL-1 40X	0.240 ± 0.010^{b}	4.671 ± 0.967^{a}
HL-2 10X	0.210 ± 0.020^{b}	3.116 ± 0.385^{a}
HL-2 20X	0.143 ± 0.035^{a}	2.976 ± 0.613^{a}
HL-2 30X	0.247 ± 0.006^{b}	2.524 ± 0.202^{a}
HL-2 40X	0.247 ± 0.006^{b}	2.309 ± 0.411^{a}

Table 3
The effect of DOW on the biomass and alcohol dehydrogenase activityof *S. cerevisiae* BCRC 21685 under subnerged fermentation.

Cultivation water	Biomass (g/100 mL)	Alcohol dehydrogenase activity (U/L)
UPW	0.157 ± 0.021^{a}	3.363 ± 0.171^{a}
TT-1 10X	0.190 ± 0.010^{b}	3.347 ± 0.318^{a}
TT-1 20X	0.170 ± 0.017^{ab}	2.976 ± 0.829^{a}
TT-1 30X	0.193 ± 0.012^{b}	2.523 ± 0.285^{a}
TT-1 40X	0.230 ± 0.017^{c}	3.009 ± 0.207^{a}
HL-1 10X	0.203 ± 0.015^{b}	3.133 ± 0.112^{b}
HL-1 20X	0.200 ± 0.010^{b}	2.985 ± 0.295^{b}
HL-1 30X	0.200 ± 0.010^{b}	2.293 ± 0.596^{a}
HL-1 40X	0.210 ± 0.026^{b}	2.836 ± 0.164^{ab}
HL-2 10X	0.160 ± 0.026^{a}	3.091 ± 0.131^{ab}
HL-2 20X	0.140 ± 0.052^{a}	3.058 ± 0.286^{ab}
HL-2 30X	0.167 ± 0.021^{a}	2.869 ± 0.214^{a}
HL-2 40X	0.163 ± 0.012^{a}	2.902 ± 0.247^{a}

the culture medium before and after fermentation to understand the yeast's absorption of minerals (Table 4). Initially, *S. cerevisiae* BCRC 23144 in the TT-1 medium primarily absorbed P and Zn, showing no significant uptake of other minerals. For *S. cerevisiae* BCRC 21833, this strain exhibited strong absorption of Mg in TT-1. However, the most noticeable reaction was with *S. cerevisiae* BCRC 21685 in TT-1 DOW, where elements such as Ca, Mg, K, Na, P, and Zn were significantly reduced post-fermentation, indicating a high demand for these minerals by this yeast strain. Overall, different yeast strains exhibited various patterns of mineral and trace element absorption and utilization in DOW as shown in Table 4. These findings provide insights into microbial interactions with their environment and reveal how to maximize the utilization of resources in DOW.

Table 4
Mineral absorption by different yeast biomass in TT-1 DOW mineral solution.

Minerals	Minerals absorption (mg/L)		
	S. cerevisiae BCRC 23144	*S. cerevisiae* BCRC 21833	*S. cerevisiae* BCRC 21685
Ca	-[a]	11.6	36
Mg	-[a]	40.4	55.1
K	-[a]	-[a]	27.2
Na	-[a]	-[a]	15.5
P	12	0.6	8.6
Zn	9.5	5.9	4.3
Fe	0.16	0.04	0.08
Mn	0.006	0.0006	0.0024
Cu	0.00227	0.0019	0.0021
Mo	0.02387	0.0224	0.022

[a] under detection limit.

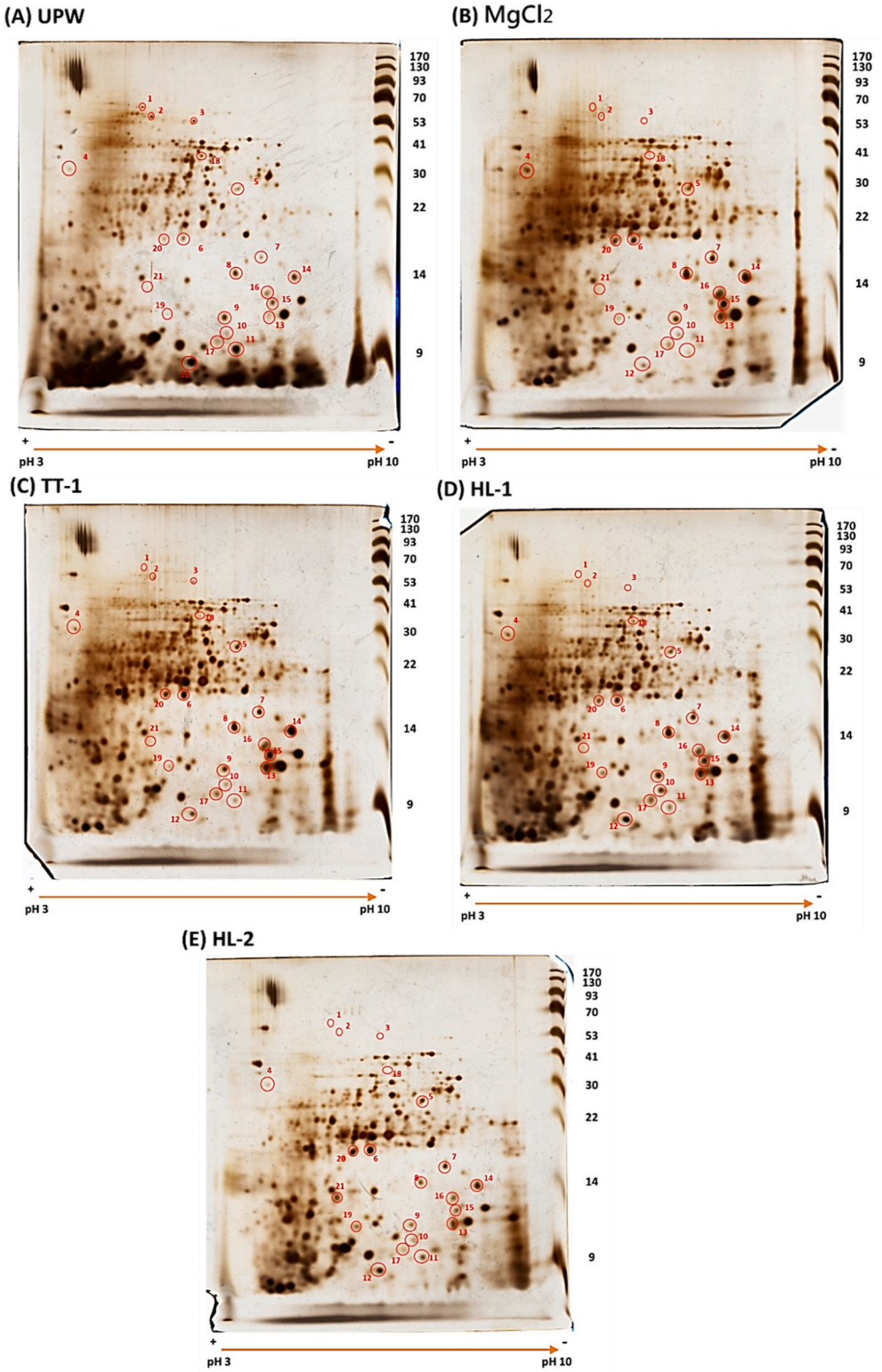

Fig. 1. Two-dimensional protein electrophoresis analysis was conducted on *S. cerevisiae* BCRC 21685 cultured in different conditions: Ultrapure Water (UPW) (A), $MgCl_2$ solution (B), and various DOW TT-1 (C), HL-1 (D), and HL-2 (E).

3.3. The effect of various DOW on protein spot expression in S. cerevisiae BCRC 21685

This study further utilized proteomics to analyze the impact of DOW on the protein expression of *S. cerevisiae* BCRC 21685 during culture. A 10X concentration served as the baseline for comparing the effects of ultrapure water (UPW), TT-1, HL-1, HL-2, and $MgCl_2$ DOW on the 2D proteomic profiles and protein spot expressions of *S. cerevisiae* BCRC 21685. Fig. 1 displays the 2D profiles for each group, from which protein spots exhibiting significant differences in expression across DOW treatments were selected for targeted identification. Based on expression differences, the gel with the highest expression was chosen for spot identification, yielding 21 target protein spots, numbered 1–21. Following LC-MS/MS identification, the proteins were identified in sequence as: Ribosome-associated molecular chaperone SSB1, Transcriptional modulator WTM1, Pyruvate decarboxylase isozyme 1, Protein disulfide-isomerase, Enolase 1, Enolase 2, Glyceraldehyde-3-phosphate dehydrogenase 3, Alcohol dehydrogenase 1, Elongation factor 2, Dihydrolipoyl dehydrogenase, and others as shown in Table 5. These proteins were categorized into four groups: those related to glycolysis and energy metabolism regulated by DOW, including Glyceraldehyde-3-phosphate dehydrogenase, Enolase, and Pyruvate kinase; those related to alcohol metabolism regulated by DOW, including pyruvate decarboxylase and alcohol dehydrogenase; and growth regulatory and other functional proteins, including Protein disulfide-isomerase and Dihydrolipoyl dehydrogenase.

In our study, we analyzed changes in protein expression levels of *S. cerevisiae* BCRC 21685 under different conditions, including three types of DOW collected from the east of Taiwan (TT, TO, KL) and treatments containing $MgCl_2$. In Fig. 2, UPW served as the control group, with expression levels set to 100 % as a baseline. The results showed that most protein expressions were elevated under DOW conditions compared to UPW, indicating that the mineral salts or other components in DOW may promote the expression of these proteins. Notably, Protein disulfide-isomerase expression significantly increased under TO and $MgCl_2$ conditions, suggesting a possible role for $MgCl_2$ in promoting specific protein expression. However, for Ribosome-associated molecular chaperone SSB1 and Transcriptional modulator WTM1, expression levels under all conditions, including $MgCl_2$, were lower than UPW, suggesting $MgCl_2$ may not be the sole influencing factor. Additionally, Elongation factor 2 expression was lower than the control under all test conditions, indicating a potential inhibition of its expression. Dihydrolipoyl dehydrogenase showed particularly high expression under the KL condition, while being less consistent under others. Overall, besides $MgCl_2$, there may be other components in DOW that promote or inhibit the expression of certain proteins. These results suggest that a comprehensive understanding of how DOW affects microbial metabolism requires further analysis of its components. Such analyses could reveal key mineral salts or components in DOW, other than $MgCl_2$, which may affect protein expression through different mechanisms.

4. Discussion

DOW, with its unique mineral composition, has been proven to enhance the fermentation effects and biological activity of various microorganisms. Specifically, when used for cultivation, the fermentation products of Antrodia cinnamomea showed stronger protective effects against thioacetamide-induced hepatic fibrosis, indicating that DOW may enhance the biological activity of this fungus or stimulate the production of specific beneficial metabolites. Additionally, DOW has been found to increase the production of monascin (MS) and ankaflavin (AK) in *Monascus* fermentation products, likely due to the positive effects of specific minerals or elements in DOW on Monascus metabolic activities. Moreover, the growth of doscorea produced by Monascus sp. was improved in DOW cultures, possibly because the mineral-rich environment provided by DOW aids in fungal growth and metabolism. Lastly, the ability of

Table 5
Identification results and expression level changes of proteins in *S. cerevisiae* BCRC 21685 cultured in different DOW.

Spot No.	Accession	protein name	Relative abundance (%)	Coverage [%]	Score Mascot	MW (kDa)	calc. pI
1	P11484	Ribosome-associated molecular chaperone SSB1	50.0	26	1411	66.6	5.44
2	Q12363	Transcriptional modulator WTM1	91.9	35	2183	48.4	5.36
3	P06169	Pyruvate decarboxylase isozyme 1	86.7	20	807	61.5	6.19
4	P17967	Protein disulfide-isomerase	100.0	19	697	58.2	4.53
5	P00924	Enolase 1	48.8	25	2255	46.8	6.62
6	P00925	Enolase 2	19.5	16	564	46.9	6
7	P00359	Glyceraldehyde-3-phosphate dehydrogenase 3	77.23	32	3397	35.7	6.96
8	P00359	Glyceraldehyde-3-phosphate dehydrogenase 3	37.7	27	1479	35.7	6.96
9	P00330	Alcohol dehydrogenase 1	67.9	34	3514	36.8	6.68
10	P00925	Enolase 2	26.5	14	1234	46.9	6
11	P32324	Elongation factor 2	22.7	3	532	93.2	6.32
12	P00359	Glyceraldehyde-3-phosphate dehydrogenase 3	58.9	11	1187	35.7	6.96
13	P00360	Glyceraldehyde-3-phosphate dehydrogenase 1	20.9	24	462	35.7	8.28
14	P00549	Pyruvate kinase 1	69.5	21	3916	54.5	7.68
15	P00360	Glyceraldehyde-3-phosphate dehydrogenase 1	50.7	21	1251	35.7	8.28
16	P00359	Glyceraldehyde-3-phosphate dehydrogenase 3	63.0	30	862	35.7	6.96
17	P00330	Alcohol dehydrogenase 1	65.8	21	953	36.8	6.68
18	P00925	Enolase 2	35.1	40	2246	46.9	6
19	P00330	Alcohol dehydrogenase 1	63.2	20	886	36.8	6.68
20	P00924	Enolase 1	20.4	16	779	46.8	6.62
21	P09624	Dihydrolipoyl dehydrogenase, mitochondrial	100	45	2378	54	8.03

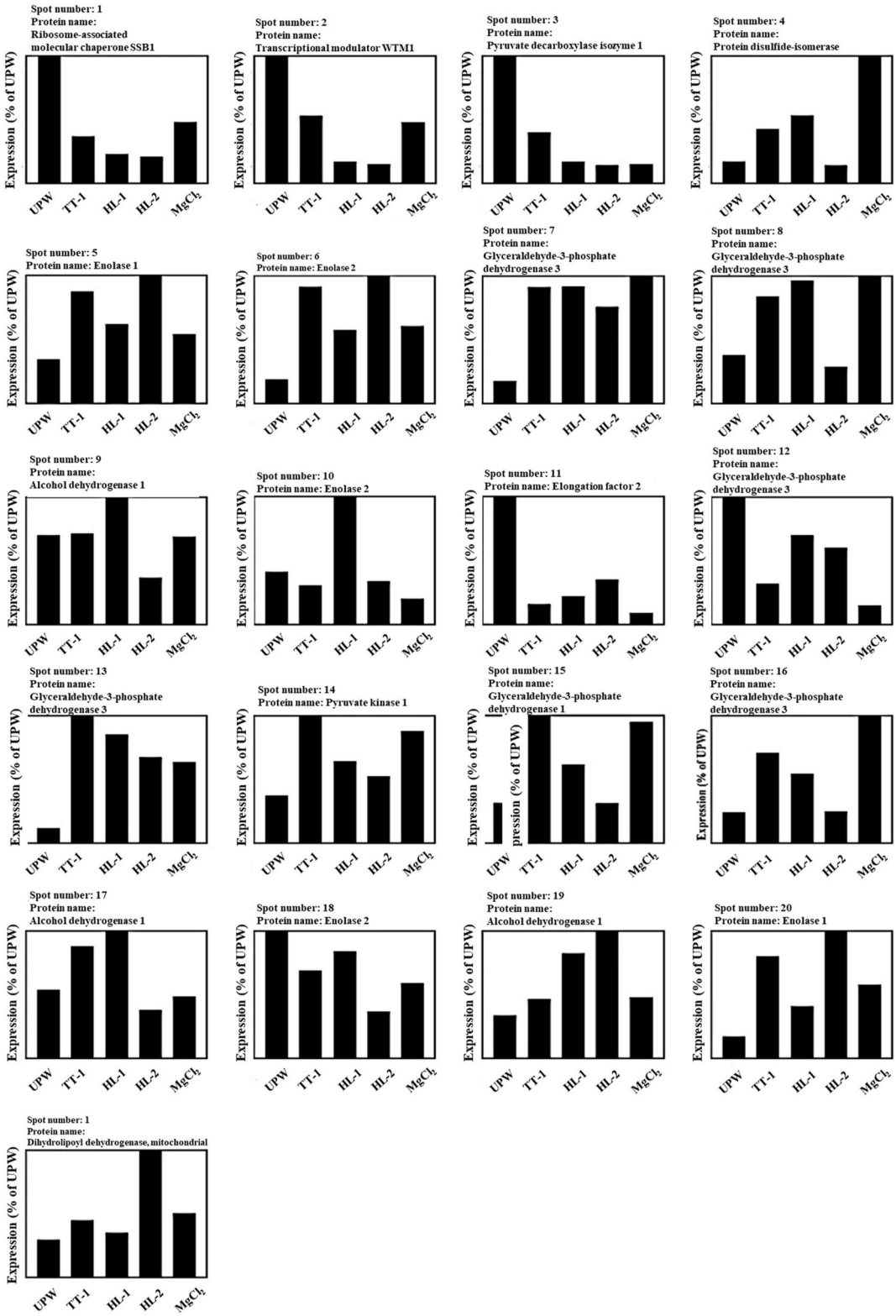

Fig. 2. Changes in protein expression levels of *S. cerevisiae* BCRC 21685 cultured in different DOW.

Antrodia cinnamomea fermentation products, supplemented with DOW, to combat Aβ-induced neurotoxicity was enhanced, which might be due to the components in DOW promoting fungal metabolic activities, thereby improving its neuroprotective effects. Hence, DOW, with its abundance of minerals and specific components, has proven to enhance the fermentation effects and biological activity of microorganisms, making it a promising culture medium for the production of microbial products with higher therapeutic and health benefits [6]. Further research has amalgamated the health benefits of DOW and its applications in fermentation biotechnology. These studies have summarized the impact of DOW on the development of fermented functional foods and the improvement of health functionalities [6]. It has been demonstrated that certain concentrations of DOW significantly enhance the alcohol dehydrogenase activity in different yeast strains, such as *S. cerevisiae* BCRC 23144 and BCRC 21685, while no notable increase in alcohol dehydrogenase activity was observed in *S. cerevisiae* BCRC 21833. These results underscore the potential application of DOW in the fermentation process and indicate that different yeast strains may respond differently.

The results of this study suggest that the absorption of minerals from DOW by yeast can impact their growth and enzyme activities. The data showed that under various dilutions of TT-1 DOW (10X, 20X, 30X, 40X), the growth and enzyme activities of three *S. cerevisiae* strains (BCRC 21685, BCRC 21833, BCRC 23144) were differently affected. These strains exhibited varying needs and absorption rates for minerals. *S. cerevisiae* BCRC 23144 showed relatively higher absorption of phosphorus and zinc, particularly phosphorus, with an absorption rate reaching 12 mg/L, significantly higher than the other two strains. However, its needs for calcium, magnesium, potassium, and sodium were minimal. *S. cerevisiae* BCRC 21833 absorbed calcium and magnesium, with a notably high absorption rate of magnesium (40.4 mg/L), but displayed poor phosphorus absorption (only 0.6 mg/L). This indicates its effectiveness in absorbing certain minerals, like magnesium, over others like phosphorus. *S. cerevisiae* BCRC 21685 exhibited higher absorption rates for almost all tested minerals, especially calcium (36 mg/L) and magnesium (55.1 mg/L). It was also the only strain that absorbed measurable amounts of potassium and sodium. This might imply that *S. cerevisiae* BCRC 21685 possesses a strong multi-mineral absorption capability. In summary, *S. cerevisiae* BCRC 21833 is particularly effective in magnesium absorption, while *S. cerevisiae* BCRC 23144 excels in the absorption of phosphorus and zinc. This information is crucial for understanding the potential of these yeast strains in various applications, such as utilizing DOW minerals for the production of nutritional supplements. Significant differences exist among yeast strains in their absorption capabilities for specific minerals. *S. cerevisiae* BCRC 21685 demonstrates superior ability in absorbing most minerals, especially sodium and potassium, which are typically challenging for microbes to absorb.

The mineral absorption capability of yeast offers multifaceted development potential in the fields of health foods and biotechnology. Firstly, in the domain of mineral-fortified foods, yeasts can enrich specific minerals such as calcium, magnesium, and zinc, making them ideal mineral-rich food additives. Such fortified yeasts can be added to bread, beverages, and other foods to enhance their nutritional value. Studies indicate that bread made with sourdough can improve the bioavailability of minerals in whole wheat flour [15]. In terms of bioavailability, yeast-enriched minerals might have higher bioavailability than chemically synthesized minerals. For example, a clinical assessment on healthy male volunteers determined that zinc from yeast supplements, when presented in an organic form within the yeast, was better absorbed [16]. Previous studies have shown that yeast cells can absorb mineral ions from the environment and accumulate them in cellular structures, such as the yeast's bioaccumulation process of selenium involving extracellular binding by a ligand assembled in the cell membrane, and intracellular accumulation associated with ion transport across the cytoplasmic membrane. In the intracellular metabolism of selenium, processes such as oxidation, reduction, methylation, and synthesis of selenoproteins are involved, for instance, in detoxification processes, allowing yeast to survive in high selenium concentration conditions. Selenium yeast helps reduce dietary selenium deficiency [17] Kieliszek et al., 2015. As a multifunctional microorganism, yeast has shown its application value in many areas.

In the yeast fermentation process, the role of magnesium ions (Mg^{2+}) is crucial. Studies have shown that the concentration of magnesium in yeast extracts directly affects ethanol fermentation performanc e, as glucose consumption and cell growth are typically coupled and especially important in the yeast fermentation process [18] Pancholi 2001. Magnesium limitation can lead to a decrease in the ethanol production rate of cell protein at the beginning of fermentation, affecting the overall ethanol yield [19] Dombek and Ingram 1986. Yeast cells absorb and accumulate Mg^{2+} from the growth medium, indicating a need for Mg^{2+}, and this accumulation is associated with the time cells enter the stationary growth phase and the maximum ethanol yield is reached [20]. Additionally, magnesium is very important for physiological protection of yeast cells against ethanol and temperature-induced stresses, with studies hypothesizing that magnesium protects cells by preventing increased cell membrane permeability caused by these stresses [21]. In beer fermentation, the accumulation of magnesium by yeast cells is considered beneficial for improving product quality and production efficiency [22]. Lastly, magnesium and calcium are crucial for enhancing yeast ethanol tolerance, helping maintain a higher CO_2 production rate at high ethanol concentrations [23] Ciesarova et al., 1996. In conclusion, these studies highlight the key role of magnesium in maintaining yeast cell metabolic activity and growth, enhancing ethanol fermentation efficiency, and strengthening stress responses. Besides magnesium ions, previous studies have indicated that calcium ions affect the morphology of the yeast Candida albicans, impacting cell wall structure and stability, thereby influencing yeast growth. Additionally, calcium balance and signaling systems in yeast cells play a key role in their growth. Calcium ions are important in maintaining cellular physiological functions and supporting cell growth. The high absorption rates of *S. cerevisiae* BCRC 21685 for these minerals may reflect its efficiency in maintaining intracellular ionic balance and promoting metabolic activities. Further, *S. cerevisiae* BCRC 21685 is the only strain to show absorption of potassium and sodium, with absorption rates of 27.2 mg/L and 15.5 mg/L, respectively. Potassium is the main cation for maintaining intracellular environmental stability, and sodium absorption may be related to the cell's adaptability to high salt environments. This finding suggests that *S. cerevisiae* BCRC 21685 might possess strong adaptability to adverse conditions. In phosphorus absorption, *S. cerevisiae* BCRC 21685 displayed a lower absorption rate compared to *S. cerevisiae* BCRC 23144 but was still higher than *S. cerevisiae* BCRC 21833. Phosphorus is a key element in the composition of biological molecules like DNA, RNA, and ATP, and the phosphorus absorption performance of *S. cerevisiae* BCRC 21685 may reflect its overall energy metabolism efficiency. *S. cerevisiae*

BCRC 21685 also showed stable absorption capabilities for trace elements like zinc, iron, manganese, copper, and molybdenum. These elements are vital for yeast growth and enzyme activities. Particularly zinc and iron, which are the active centers of many important enzymes, while manganese, copper, and molybdenum play roles in antioxidant protection and electron transfer processes, microbial interactions with these trace elements. These trace metals play key roles in development, growth, and metabolism processes, participating in various metabolic processes by acting as co-factors for enzymes or providing structural support to proteins [24] Pajarillo et al., 2021. In summary, the mineral absorption pattern of *S. cerevisiae* BCRC 21685 demonstrates its high adaptability to the DOW environment and potential metabolic advantages. These findings provide valuable insights into understanding the physiological responses of yeast in mineral-rich environments and may have practical significance for optimizing DOW fermentation processes and improving product quality. Future research should further unveil the molecular mechanisms behind these mineral absorption differences and how they affect yeast growth and production efficiency, bringing new guidance to the field of fermentation science and industrial applications.

Glycolysis, the primary pathway for cellular utilization of glucose, occurs in the cytosol and primarily produces pyruvate and ATP, thereby advancing the Krebs cycle and the electron transport chain, leading to a series of energy metabolic reactions [25]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) participates in the fifth step of glycolysis, catalyzing the conversion of glyceraldehyde 3-phosphate to 1,3-Bisphosphoglycerate and resulting in the production of NADH. GAPDH serves multiple functions within the cell, including roles in eukaryotic metabolism [26] Seidler 2013, vesicle production [27], protein regulation [28], cell cycle and apoptosis [29] Tarze et al., 2007, and protection against reactive oxygen species (ROS) [30]. Proteomic identification analysis revealed that 6 out of 21 protein spots, which differed from the UPW group, were identified as GAPDH, confirming that DOWs TT-1 and HL-1 effectively promote glycolysis, aiding microbial growth. This result is in line with previous biomass analysis. Enolase participates in the ninth step of glycolysis, converting 2-Phosphoglyceric acid (2 PG) to Phosphoenolpyruvate (PEP). This enzyme's cofactors are primarily Mg^{2+}, with other divalent metal cations including Zn^{2+}, Mn^{2+}, and Co^{2+} also serving as cofactors. Structural changes induced by metal ions binding at the active site facilitate enzyme activation [31]. Experimental results showed that 5 out of 21 protein spots, differing from the UPW group, were identified as enolase. Quantitative analysis of protein expression revealed increased expression in microorganisms cultured in DOW. While Enolase1 and Enolase2 share 95 % structural similarity and both promote glycolysis, Enolase 2 is essential, whereas Enolase is not [32]. ENO1, a magnesium-dependent enzyme, participates in glycolysis, converting 2-phosphoglycerate to phosphoenolpyruvate [18] Pancholi 2001. Pyruvate kinase, involved in the final step of glycolysis, primarily catalyzes Phosphoenolpyruvate (PEP) to produce one molecule of pyruvate and one molecule of ATP. Compared to the UPW group, cultures in various DOW groups and $MgCl_2$ group showed increased protein expression. Pyruvate kinase is a magnesium-dependent enzyme, its activity regulated by magnesium ions [33].

In summary, compared to the pure water group, the DOW groups from Taiwan and the $MgCl_2$ group showed varying degrees of

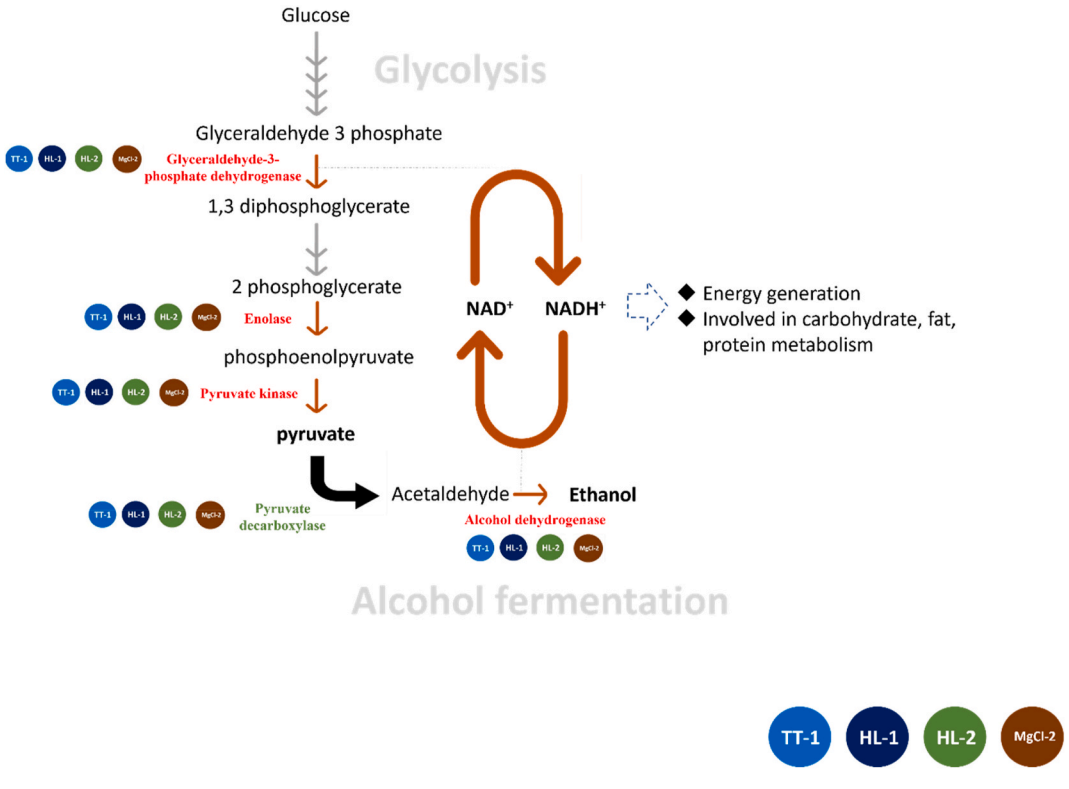

Fig. 3. Overview of the potential regulatory mechanisms influencing the growth of *S. cerevisiae* BCRC 21685 cultured in different DOW.

enhancement in glycolytic metabolic enzyme regulation (Glyceraldehyde-3-phosphate dehydrogenase, Enolase, and Pyruvate kinase), in the order of TT-1, $MgCl_2$, HL-1, HL-2. Regarding the influence of DOW on the expression of yeast alcohol metabolism regulatory proteins, the alcohol metabolism process primarily occurs anaerobically, with the glycolytic end product pyruvate further catalyzed by two key enzymes, pyruvate decarboxylase and alcohol dehydrogenase, to produce the final product, alcohol [34] Strommer and Garabagi 2009. Results showed that compared to the UPW group, the protein expression of pyruvate decarboxylase was significantly higher in other test groups, with TT-1 and HL-2 showing slightly higher expressions of yeast alcohol dehydrogenase. Alcohol dehydrogenase, a zinc-dependent protein, is closely related to yeast alcohol dehydrogenase activity [35]. This study's results indicate a positive correlation between yeast zinc ion absorption and alcohol dehydrogenase activity.

Regarding the influence of DOW on yeast energy generation and metabolism. In biological redox reactions, NADH serves as a donor of hydrogen and electrons, while NAD^+ serves as an acceptor, participating in physiological processes such as respiration, photosynthesis, and alcohol metabolism. NADH, not only a crucial coenzyme in aerobic respiration, also carries a significant amount of energy. It is primarily involved in the metabolism of carbohydrates, fats, and proteins, as NADH activates multiple enzyme systems, promoting the synthesis and metabolism of nucleic acids, proteins, polysaccharides, increasing substance turnover and regulatory control, thus closely related to improving metabolic functions. Therefore, it has a key influence on cell regeneration, repair, and protection [36]. As shown in Fig. 3, *S. cerevisiae* BCRC 21685 cultured in various DOWs can activate the enzyme activities of GAPDH and ADH, thereby generating NADH and NAD+, promoting energy generation and participating in related metabolic activities during growth.

Protein disulfide-isomerase (PDI) primarily promotes protein folding by catalyzing the isomerization of disulfide bonds in proteins. However, this enzyme is associated with calcium ions. In brewing yeast, PDI is encoded by essential genes, and the deletion of nearly a third of its C-terminal residues changes PDI's cellular localization but does not affect cell viability. Further genetic defects, however, lead to cell death [37] LaMantia and Lennarz 1993. Analysis results showed that compared to the UPW group, the protein expression of HL-2 decreased, while TT-1, HL-1, and $MgCl_2$ groups showed increased expression. Dihydrolipoyl dehydrogenase (DLD), a mitochondrial enzyme, plays a vital role in eukaryotic energy metabolism. It is essential for filamentous growth and participates in the G1/S phase of the cell cycle, with gene defects leading to cell death [38]. The enzyme is central to energy metabolism, often functioning in complex forms in organisms [39], and serves as part of mitochondrial pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (α-KGDH) complexes, involved in several physiological metabolic pathways, and associated with severe mitochondrial diseases [40]. Compared to the UPW group, all DOW test groups and the $MgCl_2$ group showed an increase in the expression of this protein.

In this study, yeast cells absorbed key minerals from DOW, including calcium (Ca, 36 mg/L), magnesium (Mg, 55.1 mg/L), potassium (K, 27.2 mg/L), sodium (Na, 15.5 mg/L), phosphorus (P, 8.6 mg/L), zinc (Zn, 4.3 mg/L), iron (Fe, 0.08 mg/L), manganese (Mn, 0.0024 mg/L), copper (Cu, 0.0021 mg/L), and molybdenum (Mo, 0.022 mg/L). The absorption of these minerals is crucial for yeast growth and proteomic regulation. Minerals such as magnesium and zinc are essential for the activity of enolase, serving as cofactors for this enzyme and crucially catalyzing key steps in the glycolysis process. This enhanced process might be related to the high concentration absorption of these minerals in DOW. The presence of calcium is also crucial for the activity of many intracellular enzymes, including those involved in cell signaling and cell wall stability. Hence, calcium absorption may play a role in maintaining yeast growth and metabolic activity. Furthermore, although the absorption of iron and manganese is low, they are essential components of many cellular enzymes, especially those involved in redox reactions and electron transport chains, vital for energy metabolism and ATP production. Overall, the absorption of these minerals has a potential regulatory effect on the proteomic expression of yeast, especially affecting enzymes involved in glycolytic metabolic pathways and energy production processes. The minerals in DOW not only support the activity of these key enzymes but also provide essential trace elements for growth, playing a significant role in enhancing the overall metabolic efficiency and growth of yeast. Therefore, the absorption of minerals in DOW is one of the key factors in regulating yeast proteomic expression and maintaining metabolic activity.

In the present study, we utilized batch fermentations to investigate the effects of different sources of DOW on yeast biomass growth and proteomic responses. However, one of the main limitations of this approach is the absence of growth kinetics measurements, which restricts the ability to dynamically assess the metabolic responses of yeast to various DOW media during fermentation. This limitation was primarily due to constraints in experimental equipment and scale. Recognizing these limitations, future research plans are already in place to expand the scope and depth of our investigations. We intend to integrate the measurement of growth kinetics in subsequent studies, employing laboratory-scale fermenters. This enhancement will allow for a detailed analysis of how specific minerals found in DOW or synthetic water affect yeast metabolism, including the expression of target proteins and enzymes. Implementing these measures will provide a more comprehensive understanding of yeast growth dynamics, substrate utilization, and metabolic product formation under different DOW conditions.

Three types of DOW have shown effectiveness in enhancing the biomass of different yeast strains. TT-1 and HL-2 effectively enhanced the SOD-like activity of yeast strain BCRC 21469. TT-1 and HL-1 effectively increased the alcohol dehydrogenase activity of yeast strain BCRC 23144. TT-1 and HL-1 effectively increased the biomass production of yeast strain BCRC 21685, but did not enhance alcohol dehydrogenase activity. Further proteomic analysis of DOW on the cultivation of *S. cerevisiae* BCRC 21685 revealed the impact on protein expression during the process. A 10X concentration was used as a baseline for comparing ultrapure water, TT-1, HL-1, HL-2, and $MgCl_2$ on the post-fermentation proteomic 2D profiles and protein spot expressions of *S. cerevisiae* BCRC 21685. The addition of DOW primarily enhanced the expression of key enzymes involved in glycolytic metabolism and regulated alcohol dehydrogenase expression. By increasing the expression of enzymes related to protein synthesis and folding, a higher biomass production was achieved.

Informed consent statement

Not applicable.

Data availability statement

All data included in this study are available upon request by contacting the corresponding author.

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CRediT authorship contribution statement

Chin-Feng Liu: Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xuan-Fang Zhang:** Investigation, Formal analysis, Data curation, Conceptualization. **Tsai-Luen Yu:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Chun-Lin Lee:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Investigation, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare no conflict of interest in this paper.

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