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Selection and proteomic profiles analysis of yeast strains isolated from traditional fermented pork (Nanx Wudl) for single cell protein production

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ABSTRACT

The increase in human population has led to imminent pressures to develop new edible proteins with decreased environmental footprints. The most promising approach involves the production of single cell protein (SCP) from yeasts, which have been utilized in a wide variety of foods for thousands of years. In this study, 102 yeast strains isolated from traditional fermented pork (Nanx Wudl) were investigated for their potential as SCP producer for the first time. Based on preliminary screening, *Saccharomyces cerevisiae* Y70 and *Candida parapsilosis* H5Y13, both showing high protein content and excellent growth capability, were selected for further analysis *via* 4D-DIA proteomics technology. Proteomic analysis indicated that the oxidative metabolism pathways, including TCA cycle, oxidative phosphorylation and pentose phosphate pathway, may have a significant impact on global protein synthesis and production. This study provides useful information for selecting SCP-producing yeast from Chinese fermented meat products and contribute to a deeper understanding of the underlying metabolic mechanisms behind global protein synthesis in yeast. Furthermore, these findings also provide potential molecular targets for genetic engineering modifications in yeast, aimed at constructing highly efficient cell factories for protein production.

1. Introduction

In recent years, there has been a growing interest in single cell protein (SCP), which refers to edible unicellular microorganisms, owing to their highly efficient substrate conversion and productivity[1]. SCP contains a substantial quantity of protein, comprising approximately 60%–82% of its dry cell mass by weight[2]. It is particularly noteworthy for its rich content of essential amino acids, such as lysine and methionine, which are often limited in most plant and animal sources[3]. Additionally, SCP can provide other essential nutritional components, including carbohydrates, fats, vitamins, and minerals[4]. SCP technology has emerged as a promising solution to alleviate the global protein food shortage. One of its significant advantages is its independence from climatic and seasonal variations, soil characteristics, and land availability[5]. This feature makes SCP production more effective compared to traditional protein sources that are significantly influenced by environmental factors. Through the use of SCP technology, it becomes possible to overcome the limitations imposed by these factors and secure a stable and sustainable protein supply[6-7]. Moreover, SCP technology offers an additional environmental benefit compared to traditional protein sources. Unlike plant-based and animal-based protein production, SCP technology does not emit greenhouse gases into the environment. This is due to the fact that SCP is typically produced through controlled fermentation processes that do not involve the release of carbon dioxide or other greenhouse gases[8]. By utilizing SCP as a protein source, we can

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not only address the global protein shortage but also contribute to mitigating climate change by reducing our environmental footprint[9]. SCP has gained significant recognition in both animal feed and food applications. It finds extensive use in the food industry as a versatile ingredient, serving as meat substitutes, texturizing agents, flavor enhancers, vitamin carriers, emulsifiers, and enhancers of the nutritive value of food products. Its wide range of applications highlights its potential to address various needs within the food industry, from improving product texture and taste to enhancing nutritional content. SCP's versatility and diverse functionality make it a valuable ingredient in food production[10]. Currently, SCP-based food is commercially produced under various brand names, each catering to specific applications and market segments. Some of the well-known commercial names for SCP include Quorn®, AlgaVia®, Marmite®, Vitam-R®, Pruteen®, Brovile®, and FermentIQ™. These brands offer a diverse range of SCP products, each with its unique characteristics and functionalities. The availability of SCP under different commercial names provides consumers and industries with a wide selection of options to meet their specific needs and preferences[11]. With the projected global population reaching 9 billion by 2050, there is compelling evidence indicating that conventional agricultural methods may be inadequate to meet the demand, thereby posing a significant risk of food shortage. Autotrophic SCP offers a fail-safe option for mass food production, capable of reliably generating food even under harsh climate conditions[12-15].

The widespread application of SCP for human consumption is constrained by 2 critical factors: high nucleic acid content and poor cell wall digestion. These factors significantly limit the nutritional value of SCP[16]. It is essential to choose high-quality microbial strains for the production of SCP due to their significant impact on production efficiency, quality, and cost. Consequently, the screening and enhancement of microbial strains will be critical for the future advancement of SCP production. Yeast protein is nutritionally rich and contains a wide variety of amino acids. The large size of the yeast cells makes them suitable for fermenting a variety of industrial waste, by-products, food residues, and meat products to produce SCP)[17]. Various yeast species exhibit notable variations in protein content when fermenting diverse waste substrates. For instance, *Candida utilis* produces protein content of 26% when fermenting salad oil wastewater, while fermenting shrimp shell waste results in a high protein content of 70%. *Saccharomyces cerevisiae* yields protein content differing by 20% when fermenting distillery sludge compared to fermenting molasses, with values of 33% and 53% respectively[18-21].

In this study, a total of 103 yeast strains were isolated from traditional Chinese Dong fermented pork (Nanx Wudl) originating from 6 distinct regions. Nanx Wudl is a naturally fermented pork delicacy, primarily consumed by the Dong, Miao, and Tujia ethnic groups from Hunan and Yunnan in China[22]. In this study, we utilized yeast strains isolated from Nanx Wudl as the subject of our research. Given the abundance of yeast resources in Nanx Wudl, yeast isolates are known for their high edibility safety, pleasant flavor, and excellent growth properties, making them promising candidates for SCP production. However, there has been no prior report on the screening of SCP-producing strains from Nanx Wudl. Our study pioneers the exploration of the potential in isolating strains from traditional fermented pork (Nanx Wudl) for SCP production. We tested these isolated strains by using the protein yield and growth capability of the strains as screening criteria, we have selected strains with outstanding comprehensive performance, laying the foundation for the next step of SCP-based meat research and development work. The 2 screened strains from isolates with outstanding overall performance were further optimized and analyzed through single-factor exploration of cultivation conditions and proteomic. It was discovered that growth time is one of the most critical factors influencing protein yield. Subsequent analysis of their proteomics unveiled that these 2 strains possess robust growth regulatory capabilities, adapting to external oxidative stress by regulating the production of proteins and enzymes. Our ultimate goal was to obtain yeast strains that are suitable for the production of SCP and explore potential regulation mechanism of SCP in yeast.

2. Materials and methods

2.1 Isolation of yeast strains

A total of 15 Nanx Wudl samples in this study were collected from different manufacturers in the Dong ethnic minority regions in Hunan and Yunnan, China. These manufacturers are small-scale facilities, producing Nanx Wudl without the use of microbial starters. A total of 103 yeast strains were isolated from Nanx Wudl samples. Subsequently, the yeast strains present in Nanx Wudl were quantified by culturing on potato dextrose agar (PDA, Luqiao, Beijing, China) for 48 h at 28 °C. Following the observation of colony morphology, isolates were purified through streaking on PDA and subsequently preserved at –80 °C in yeast peptone dextrose medium (YPD, 5 g/L yeast extraction, 10 g/L peptone and 20 g/L glucose) with 30% (*V*/*V*) glycerol supplementation.

2.2 Identification of yeast strains

Internal Transcribed Spacer (ITS), 18S rDNA and 26S rDNA sequencing were applied to analyze the purified isolates. The genomic DNA was extracted *via* the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the ITS region, we amplified the sequence using universal primers ITS1 and ITS4[23], and for 18S rDNA and 26S rDNA, we utilized primers pairs NS1 and NS4, and NL1 and NL4, respectively[24]. Subsequent sequencing of the amplified products was performed by Thermo Fisher Scientific. We then compared the resulting sequences with those of type strains in the GenBank database, identifying the species based on a criterion of 99%–100% sequence match.

2.3 Optimization of cultivation condition for screening strains

After determining the carbon source, inorganic nitrogen source, and the concentration for screening purposes. We used single-factor experiments to study the effects of carbon source, nitrogen source, inorganic salts, and culture time on the growth performance and protein yield of the strains. Each group of experiments had 3 replicates. The orthogonal experiments were adopted to optimize the amounts of glucose (40, 50, 60 g/L), mixed animal-based peptone (tryptone, 15, 20, 25 g/L) produced by Oxoid Limited (Basingstoke, UK),

and culturing time (16, 20, 24 h), to determine the bestcomposition of the culture medium and time for screening purposes.

2.4 Measurement of the cell mass

Initially, we performed a primary screening based on the size of the precipitate produced by the strains in YPD medium for 48 h at 28 °C, subsequently eliminating strains with notably slower growth. Following this, the strains exhibiting superior growth were inoculated into 300 mL YPD medium in 1 000 mL flasks and cultured at 28 °C and 150 r/min for 24 h. The cultured strains were then subjected to lyophilization to remove moisture. The grown strains were collected and centrifuged in 6 000 r/min for 10 min, and washed with deionized water for 3 times. After removed the supernatant, the harvested strains were pre-freezed in –20 °C for 1 h. The frozen strains were then placed in a lyophilization facility (Epsilon 2-6D LSCplus, Martin Christ Gefriertrocknungsanlage, Osterode am Harz, Germany) for freeze-drying treatment. Then the freeze-dried strains were measured on a four-position balance to confirm their cell mass.

2.5 Measurement of the protein yield

Two methods were mainly applied in this study, bicinchoninic acid (BCA) method and Kjeldahl-method. Using Kjeldahl-method, the freeze-dried samples were weighed and placed into the special tubes, and then labeled. These tubes were added with the digestive tablets and 10 mL of 98% H_2SO_4. Next, they were digested at 420 °C on the digestive apparatus for 1 h. The distillation and titration were carried out by Kjeldahl apparatus (KT8400, FOSS, Hilleroed, Denmark) and the protein yield (protein content in dry weight) can be obtained. BCA analysis was described in supplementary method. The statistical analysis of protein content from these strains was conducted.

2.6 Proteomics analysis of screened strains

The selected strains were cultured for 16 h (exponential growth phase) and 24 h (stationary growth phase) respectively under their optimal culture condition. Subsequently, the protein was flash-frozen in liquid nitrogen and analyzed using 4D-DIA technology by Scale Biomedicine Technology Co., Ltd. (Beijing, China). Samples were dissolved in 0.1% formic acid, and 200 ng of peptides were separated using an AUR3-15075 C_{18} column. Data-independent acquisition (DIA) data was collected in the diaPASEF mode. Raw Data of DIA were processed and analyzed by Spectronaut 17 (Biognosys AG, Switzerland) with default settings. A 1.5-fold change served as the threshold for differential expression, and a statistical *t*-test with a significance threshold of $P < 0.05$ was applied.

2.7 Data analysis

All experiments were conducted in triplicate. For the determination of cultivation condition and protein yield of isolated strains, data were presented as mean values and evaluated using SPSS version 19.0 (International Business Machines, USA). Significant differences between mean values ($P < 0.05$) were determined via one-way ANOVA with Tukey's test.

3. Results and discussion

3.1 Identification of the strains isolated from Chinese Dong fermented pork

In this research, a total of 102 yeast strains were isolated from traditional Chinese Dong fermented pork (Nanx Wudl) originating from 6 distinct regions. The genetic analysis was performed based on the ITS, 18S rDNA or 26S rDNA sequences. The sequencing results and NCBI accession numbers are summarized in Table 1. These 102 identified yeast strains belonged to 14 different species within 9 genus (Fig. 1), which includes *Pichia membranifaciens*, *Pichia kudriavzevii*, *Kazachstania exigua*, *Kazachstania bulderi*, *Kazachstania naganishii*, *Candida zeylanoides*, *Candida parapsilosis*, *Candida glabrata*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *Millerozyma farinose*, *Meyerozyma guilliermondii*, and *Debaryomyces hansenii*. *K. bulderi* was the majority species, accounting for 32 strains, followed by *C. parapsilosis* (14 strains), *S. cerevisiae*

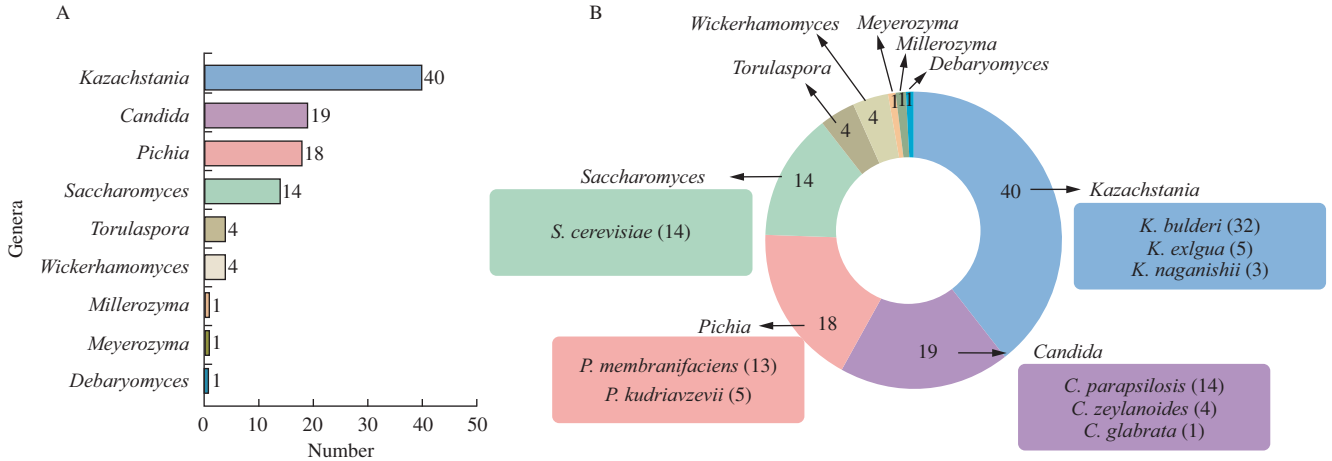

Fig. 1 Yeast strains isolated from traditional Chinese Dong fermented pork (Nanx Wudl) of 6 different regions. (A) Number of strains with each genus; (B) Isolated 14 species within 9 genera.

Table 1
Identities of the sequenced genes (ITS or 26S rDNA) of yeast strains and the accession numbers of the entries with the highest identity.

Strain	Species	Gene	Identity (%)	Accession number
H1Y18	*P. membranifaciens*	ITS	99.77	OP418412
H1Y19	*P. membranifaciens*	ITS	99.54	OP418412
H1Y20	*P. membranifaciens*	ITS	99.54	OP418412
H1Y22	*P. membranifaciens*	ITS	99.77	OP418410
H1Y23	*P. membranifaciens*	ITS	99.77	KY104622
H1Y24	*P. membranifaciens*	ITS	99.77	OP418410
H1Y25	*P. membranifaciens*	ITS	98.89	OP418412
H1Y27	*P. membranifaciens*	ITS	99.78	OP418410
H1Y28	*P. membranifaciens*	ITS	99.11	OP418410
H1Y33	*C. zeylanoides*	18S rDNA	99.49	MK203831
H2Y3	*P. membranifaciens*	ITS	99.55	OP418410
H2Y4	*K. exigua*	26S rDNA	98.28	GU138449
H2Y5	*K. exigua*	26S rDNA	100.00	KC111879
H2Y7	*K. exigua*	26S rDNA	98.80	GU138449
H2Y8	*K. bulderi*	ITS	100.00	KY103628
H2Y9	*P. membranifaciens*	ITS	100.00	OP418410
H2Y10	*K. exigua*	26S rDNA	98.63	GU138449
H2Y11	*K. exigua*	26S rDNA	98.60	GU138449
H2Y13	*K. bulderi*	ITS	99.86	KY103628
H2Y14	*K. bulderi*	ITS	99.86	KY103628
H3Y5	*K. bulderi*	ITS	99.71	KY103628
H5Y1	*M. farinosa*	ITS	99.64	MH252559
H5Y2	*C. parapsilosis*	18S rDNA	99.60	FJ515169
H5Y3	*C. parapsilosis*	18S rDNA	100.00	FJ515169
H5Y4	*C. parapsilosis*	ITS	99.46	MF979197
H5Y5	*S. cerevisiae*	ITS	99.50	CP093776
H5Y6	*S. cerevisiae*	ITS	99.50	CP093776
H5Y7	*S. cerevisiae*	ITS	98.64	KU535601
H5Y8	*C. parapsilosis*	ITS	100.00	PP033873
H5Y9	*S. cerevisiae*	ITS	98.10	OP644109
H5Y10	*C. parapsilosis*	ITS	99.31	PP033873
H5Y11	*C. parapsilosis*	ITS	99.31	PP033873
H5Y12	*C. parapsilosis*	ITS	99.60	LC317527
H5Y13	*C. parapsilosis*	ITS	99.60	LC317527
H5Y14	*S. cerevisiae*	ITS	99.38	OM281252
H5Y15	*S. cerevisiae*	ITS	99.50	MW710259
H5Y16	*C. zeylanoides*	ITS	99.32	KX385848
H5Y17	*S. cerevisiae*	ITS	98.90	MW710204
H5Y18	*C. parapsilosis*	ITS	98.97	AB109277
H5Y19	*S. cerevisiae*	ITS	99.50	CP093776
H5Y20	*S. cerevisiae*	ITS	98.77	KU535606
H5Y21	*C. zeylanoides*	ITS	99.32	KX385848
H5Y22	*P. membranifaciens*	ITS	98.87	KY104631
H5Y23	*S. cerevisiae*	ITS	98.89	CP093776
H5Y24	*P. membranifaciens*	ITS	99.32	DQ104715
H5Y25	*C. parapsilosis*	ITS	99.38	AB109277
H5Y26	*S. cerevisiae*	ITS	99.13	KU535607
H5Y27	*C. parapsilosis*	ITS	95.15	EU564209
H5Y28	*C. parapsilosis*	ITS	98.62	EU564209
H5Y29	*S. cerevisiae*	ITS	99.01	KU535607

Table 1 (*Continued*)

Strain	Species	Gene	Identity (%)	Accession number
H5Y30	*S. cerevisiae*	ITS	99.13	KU535607
H5Y31	*S. cerevisiae*	ITS	99.14	KF728774
Y70	*S. cerevisiae*	ITS	99.14	CP093776
ThY1	*D. hansenii*	26S rDNA	99.65	KT934955.1
ThY3	*C. zeylanoides*	26S rDNA	99.48	EU327106.1
A11Y1	*K. bulderi*	ITS	99.85	MT645410
A11Y2	*K. bulderi*	ITS	99.28	CP134421
A11Y3	*K. bulderi*	ITS	99.14	CP134421
A11Y4	*K. bulderi*	ITS	99.71	MT645410
A11Y5	*K. bulderi*	ITS	99.85	MT645410
A11Y6	*K. bulderi*	ITS	99.56	MT645410
A11Y7	*K. bulderi*	ITS	99.56	MT645403
A11Y8	*K. bulderi*	ITS	99.56	MT645403
A11Y9	*K. bulderi*	ITS	99.85	MT645410
A11Y10	*K. bulderi*	ITS	99.42	CP134421
A11Y11	*K. bulderi*	ITS	99.27	MT645403
A11Y12	*K. bulderi*	ITS	99.56	MT645410
A11Y13	*K. bulderi*	ITS	99.85	MT645410
A11Y14	*K. bulderi*	ITS	99.71	MT645410
A11Y15	*K. bulderi*	ITS	99.27	MT645403
A11Y16	*T. delbrueckii*	ITS	100.00	AB469378
A11Y17	*T. delbrueckii*	ITS	99.61	MN371902
A11Y18	*T. delbrueckii*	ITS	99.87	AB469378
A11Y19	*K. bulderi*	ITS	99.27	MT645403
A11Y20	*K. bulderi*	ITS	99.71	MT645410
A11Y21	*T. delbrueckii*	ITS	99.61	MN371902
A11Y22	*K. bulderi*	ITS	99.71	MT645410
A6Y1	*K. bulderi*	ITS	99.86	MT645410
A6Y2	*P. kudriavzevii*	ITS	100.00	NR131315
A6Y3	*P. kudriavzevii*	ITS	99.79	NR131315
A6Y4	*P. kudriavzevii*	ITS	100.00	NR131315
A6Y5	*K. bulderi*	ITS	99.86	MT645410
A6Y6	*P. kudriavzevii*	ITS	100.00	NR131315
A6Y7	*K. bulderi*	ITS	100.00	NR138198
A6Y8	*K. bulderi*	ITS	100.00	NR138198
A6Y9	*P. kudriavzevii*	ITS	100.00	NR131315
A6Y10	*K. bulderi*	ITS	100.00	NR138198
A6Y11	*K. bulderi*	ITS	100.00	NR138198
A6Y12	*K. bulderi*	ITS	100.00	NR138198
A6Y13	*K. bulderi*	ITS	99.86	MT645410
A6Y14	*P. kudriavzevii*	ITS	98.51	JQ425391
A6Y15	*K. bulderi*	ITS	99.86	MT645410
A7Y1	*K. naganishii*	ITS	98.19	HE978314
A7Y2	*C. glabrata*	ITS	100.00	MH628213
A7Y3	*K. naganishii*	ITS	98.18	HE978314
A7Y4	*K. naganishii*	ITS	98.19	HE978314
A7Y5	*K. naganishii*	ITS	99.19	HE978314
A8Y1	*W. anomalus*	ITS	99.83	MT136545
A8Y2	*W. anomalus*	ITS	99.83	MT136545
A8Y3	*W. anomalus*	ITS	99.47	NR111210
A8Y4	*W. anomalus*	ITS	99.83	MT136545
A8Y5	*M. guilliermondii*	ITS	99.29	NR111247

(14 strains), and *P. membranifaciens* (13 strains). While the other species respectively took up the number less than 5 strains among the 102 strains. Upon reviewing the current research status of these strains, it has been noted that they have not been identified as the suitable starter strains for the production of microbial proteins. To identify initial strains that are suitable for large-scale SCP production in the future, we screened from these strains based on their growth conditions and protein yield.

3.2 Screening of strain via cell mass and protein yield

The screening process encompassed 3 primary stages: evaluation of strain growth performance, assessment of strain SCP production levels and optimization of the cultivation conditions. Considering that the growth performance and metabolism of microorganisms are influenced by carbon and nitrogen sources, we initiated the screening of using the traditional medium YPD that contains peptone, glucose and yeast extracts for culturing the isolates from Nanx Wudl[25].

We conducted an initial screening of the growth performance of the isolated strains based on their dry weight, which can evaluate the cell mass of the strain. Based on the comparison of dry weight measurements (Fig. 2 and Table 2), the strains with superior growth mainly belonged to the following 10 species: *P. kudriavzevii*, *K. naganishii*, *K. exigua*, *K. bulderi*, *C. parapsilosis*, *C. zeylanoides*, *C. glabrata*, *W. anomalus*, *T. delbrueckii* and *S. cerevisiae*. Among them, the 3 strains belonged to *K. naganishii* exhibited the best performance, with fast growth and higher cell mass than other strains. Some species provided only 1 strain that reached top 25 of cell mass in total 102 strains, such as *C. glabrata*, *C. parapsilosis* and *C. zeylanoides*. While, some species offered more than 2 strains allowing cell mass exhibited excellent growth but with obvious variance, such as *P. kudriavzevii* and *S. cerevisiae*. These strains were next selected to conduct protein measurement. The next step will determine their protein expression levels to select potential candidates for microbial protein development.

Based on the screening results of the growth performance of 102 strains, 25 strains exhibiting the best growth performance have been initially selected for further investigation (Fig. S1). Kjeldahl method and BCA analyses were both applied to determine total nitrogen on these 25 strains for the measurement of their crude protein content. The results indicated that among various yeast species, the strains of *K. naganishii* (A7Y1, A7Y3, A7Y5) and *T. delbrueckii* (A11Y17 and A11Y18) that exhibited excellent growth performance (dry weight of 10–11 g/L) but had low protein yield (protein content 35%–42%). On the other hand, 2 strains, *S. cerevisiae* strain Y70 (dry weight 9.5 g/L, protein content 55.81%) and *C. parapsilosis* strain H5Y13 (dry weight 8.9 g/L, protein content 53.98%) after protein test using Kjeldahl method and statistics analysis, demonstrated their outstanding performance in terms of both growth performance and protein yield (Figs. 2 and 3).

Table 2
Number of strains that dry weight exceeded 1.6 g from 300 mL YPD cultured under optimal condition.

Species	Total isolate number	Dry weight > 1.6 g
P. membranifaciens	13	4
P. kudriavzevii	5	0
K. bulderi	32	2
K. exigua	5	2
K. naganishii	4	3
C. parapsilosis	14	1
C. zeylanoides	4	1
C. glabrata	1	1
S. cerevisiae	14	7
W. anomalus	4	2
T. delbrueckii	4	2
M. farinose	1	0
M. guilliermondii	1	0
D. hansenii	1	0

3.3 Optimizations of the cultivation condition impacting on cell growth and protein yield

The component and quality of biomass in the culture medium have been shown to improve SCP production[26]. The efficiency of SCP production is influenced by various factors such as substrate type, substrate concentration, availability of carbon and nitrogen sources, pH, temperature, and inoculation amount[27]. In this study, we investigate the different carbon sources, nitrogen sources, inorganic salts, temperature

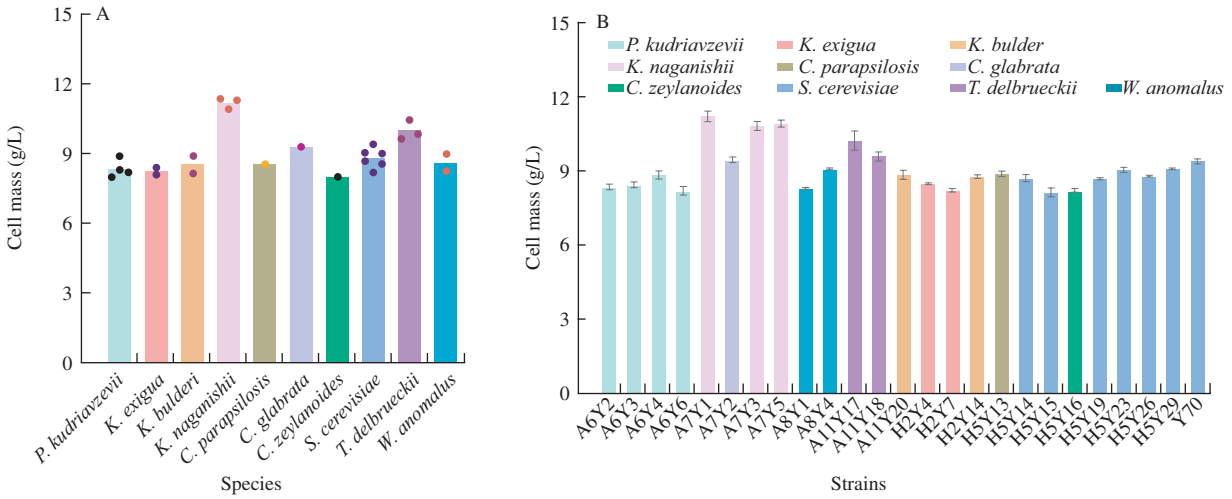

Fig. 2 Cell mass of selected strains. A total of 25 strains with high cell mass were obtained by measuring dry weight (> 1.6 g) in 300 mL medium. (A) Species of isolates with the 25 most cell mass. (B) Cell mass of top 25 isolated strains. Each vertical bar represents one strain. Bars represent mean ± SD of 3 independent samples.

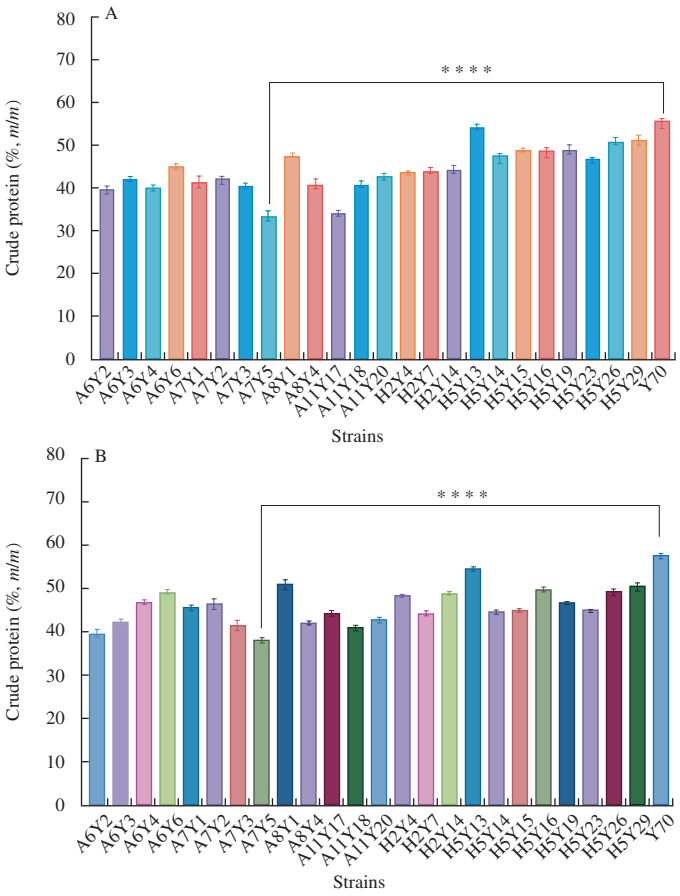

Fig. 3 Crude protein content (protein yield) of selected 25 strains. Crude protein content of each strain was determined using the Kjeldahl method and BCA. (A) Protein yield of strains (K-method). (B) Protein yield of strains (BCA). Each vertical bar represents 1 strain. Bars represent mean ± SD of 3 independent samples, ****$P < 0.000\ 1$.

and pH. Based on the results, we finally found that 50 g/L glucose as the carbon source (Fig. S2) in the cultivation system and a 20 g/L mixed-animal peptone as the nitrogen source (Fig. S3) in the basal medium YPD with supplement 1.5 g/L K2HPO4 and 1 g/L MgSO4 (Fig. S4) were the optimal condition (28 °C, pH 5.5) for *S. cerevisiae* Y70 (Figs. S5 and S6).

Due to varying requirements for the culture medium among different strains, optimizing the culture conditions is necessary to achieve the maximum single-cell protein yield for each selected strain. We selected the strain with consistent results in both BCA and Kjeldahl-method tests among the ten species initially screened to further optimize using orthogonal experiments under different conditions (Table S1). Based on the results of the single-factor experiments on the carbon source, nitrogen source, and inorganic salts, we adopted orthogonal experiments to optimize the amounts of glucose (40, 50, 60 g/L), mixed animal-based peptone (15, 20, 25 g/L), and culturing time (16, 20, 24 h), to determine the best composition of the culture medium and time for screening purposes.

The results of orthogonal test were shown in Fig. 4, and the protein yield under the optimal condition showed slightly different optimization conditions (Table 3). The optimal cultivation condition for *S. cerevisiae* strain Y70 was No. 14 (50 g/L glucose, 20 g/L animal-mixed peptone and 24 h), and the protein yield of *S. cerevisiae* strain Y70 was 56.72%, the highest among all conditions. The slight difference was that the optimal cultivation condition for *C. parapsilosis* strain H5Y13 was No.16 (60 g/L glucose, 15 g/L animal-mixed peptone and 24 h). Under this cultivation condition, *C. parapsilosis* strain H5Y13 exhibited the highest protein yield of all conditions, which is at 55.64%. Considering relatively excellent growth performance and microbial protein production, *S. cerevisiae* Y70 and *C. parapsilosis* H5Y13 were selected as the initial strains for further study of usable SCP. The results from the Box-Behnken response surface analysis indicate that, in comparison with the other 2 single factors, incubation time has the greatest impact on these 2 strains (Tables S2 and S3, Figs. S7 and S8).

Table 3
The optimized cultivation conditions of the different strains.

Species	Strains	Conditions	Protein yield (%)
P. kudriavzevii	A6Y2	No. 25	42.67
K. naganishii	A7Y5	No. 18	36.79
K. exigua	H2Y7	No. 26	45.96
K. bulderi	H2Y14	No. 17	46.22
C. parapsilosis	H5Y13	No. 16	54.89
C. zeylanoides	H5Y16	No. 20	50.98
C. glabrata	A7Y2	No. 22	47.19
W. anomalus	A8Y4	No. 15	41.77
T. delbrueckii	A11Y18	No. 14	41.55
S. cerevisiae	Y70	No. 14	55.92

Additionally, to assess the safety of the selected strains for use as food, the RNA content of *S. cerevisiae* Y70 and *C. parapsilosis* H5Y13 was measured. The fast-growing yeast typically contain a high-concentrated nucleic acid, particularly RNA. The results showed that RNA content increased proportionally with the protein expression ability of the strains but did not exceed 7% (Fig. 4C). Therefore, the screened strains can be classified as low-RNA, high-single-cell protein strains with excellent growth performance.

3.4 Analysis of the protein of two strains using 4D-DIA technology

Through optimization of culture conditions, certain factors such as nitrogen source, carbon source and culture time could affect the global protein expression of these 2 strains. However, we found that, as the cultivation process progressed, the protein expression (percentage content) did not perfectly correlate with growth status (dry weight). Also, the culturing time was showed as the most significant factor impacting on protein yield. Therefore, we conducted 4D-DIA proteomic analysis to investigate differentially expressed proteins in various growth stages. The strains Y70 and H5Y13 were cultured for 16 h (exponential growth phase) and 24 h (stationary growth phase) respectively under their optimal culture condition (Y70 was cultured in YPD containing 50 g/L glucose, 20 g/L peptone, 5 g/L yeast and 1.5 g/L H_2KPO_4 and H5Y32 was cultured in YPD containing 60 g/L glucose, 15 g/L peptone, 5 g/L yeast and 1.5 g/L H_2KPO_4). Subsequently, the protein was flash-frozen in liquid nitrogen and analyzed for proteomic analysis using Data-independent acquisition (DIA) that is a novel, comprehensive quantitative technology for proteomic analysis[28]. In this study, 4D-DIA technology was applied for quantitative proteomic analysis of the Y70 and H5Y13 strains, representing the first instance of applying this technology to analyze proteins from strains that produce SCP. In proteomics analysis for different growth stages of 2 strains, the substantial data generated through mass spectrometry encompasses all present processes and their variations within the organism. The main objective of proteomic

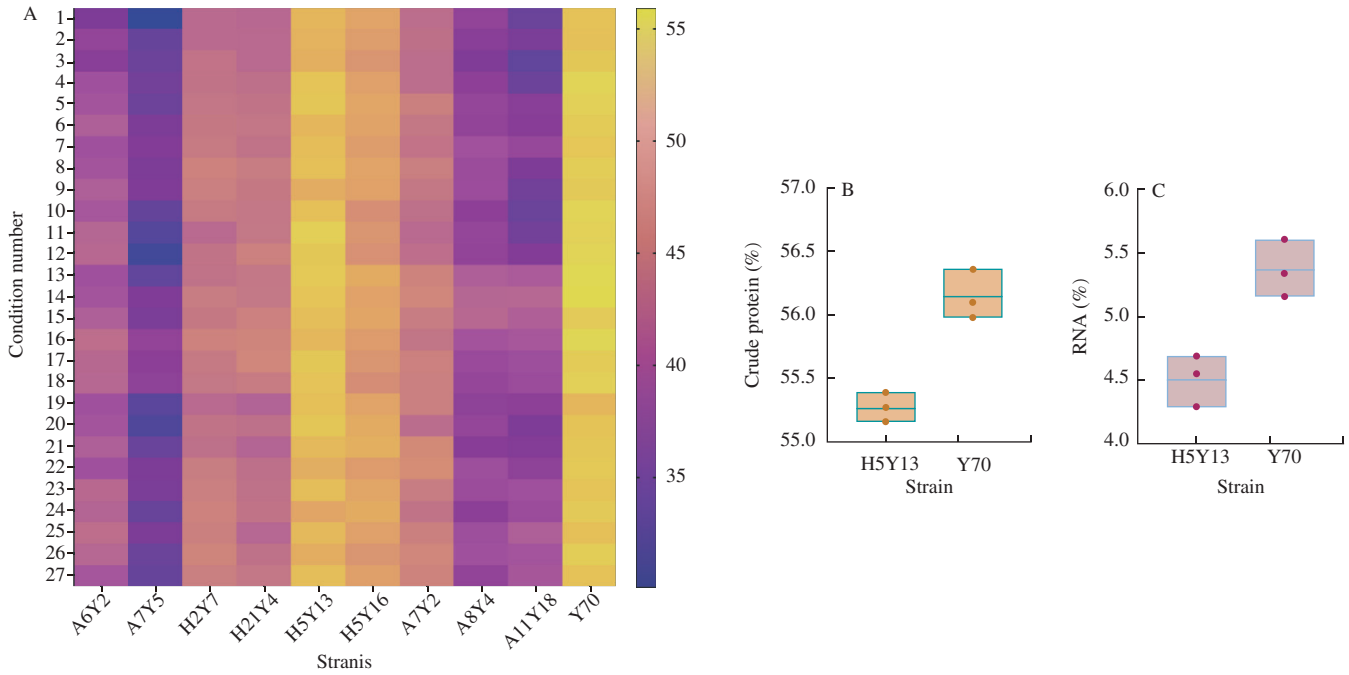

Fig. 4 By optimizing the 3 key culture factors, 2 strains with crude protein content of around 55% were picked out. (A) the optimization of conditions, a heat map of orthogonal test results. (B) Protein yield. (C) RNA content. Each point represents 1 strain. Points represent individual measurements.

bioinformatics is to identify and elucidate changes in organisms as well as the origins and underlying mechanisms of these changes from this vast and complex experimental data. Therefore, we performed an in-depth analysis of the qualitative and quantitative characteristics of proteins, encompassing Gene Ontology (GO), protein domains, Cluster of Orthologous Genes (COG) functions, and Kyoto Encyclopedia of Genes and Genomes (KEGG).

According to results of proteomic analysis, strain Y70 totally contains 4 178 proteins and 61 846 peptides. H5Y13 contains total 1 463 proteins and 7 318 peptides. A 1.5-fold change served as the threshold for differential expression, and a statistical *t*-test with a significance threshold of $P < 0.05$ was applied. In the comparison between H5Y13_24h and H5Y13_16h, 224 proteins exhibited altered expression levels, with 112 upregulated and 112 downregulated proteins. For the Y70_24h and Y70_16h comparison, 142 proteins displayed changes in expression, with 103 upregulated and 39 downregulated proteins. The volcano plot data in Figs. 5A and B showed the differential protein expression between the 16- and 24-h growth stages of strain Y70 and H5Y13. The variance in protein expression was also depicted through 2-dimensional hierarchical cluster analysis of both proteins and samples, as shown in the heatmaps (Figs. 5C and D). The proteins such as citrate synthase, 2-oxoglutarate dehydrogenase, peroxisomal catalase A, mitochondrial biogenesis regulation protein and etc. significantly upregulated the stability of strain Y70 and H5Y13. The analysis results suggest that protein regulation in cells changes during their process of growth and metabolism. Subsequently, a comprehensive bioinformatics analysis, encompassing protein function annotation, functional enrichment, and protein interaction network analysis, was conducted on the identified proteins exhibiting differential expression.

3.5 Analysis of key proteins involved in cell growth and metabolism process.

The enriched GO analysis suggested that the upregulated proteins collectively contribute to the growth and metabolism of the strains during its growth process (Figs. S9 and S10). In H5Y13, the most

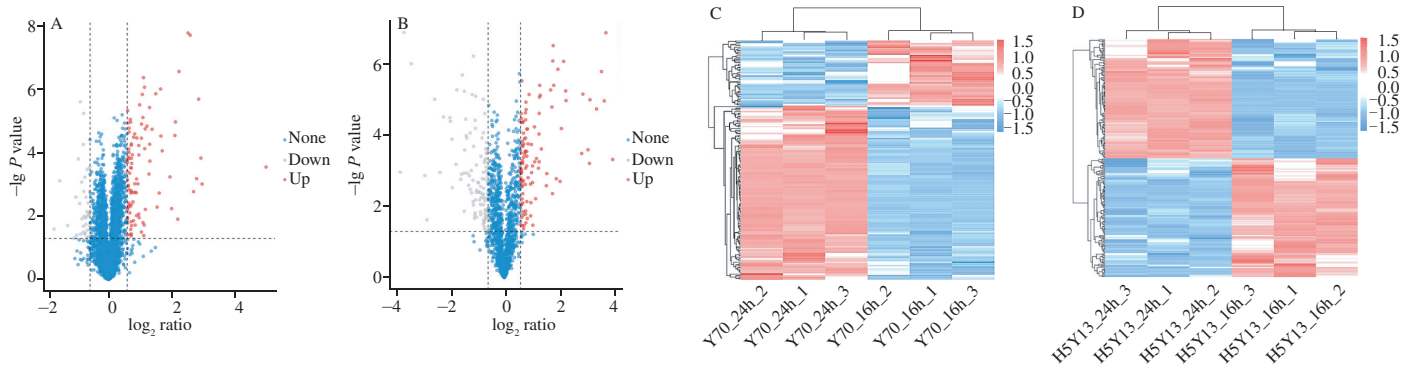

Fig. 5 Proteomics analysis of differential proteins in strain Y70 and H5Y13 in growth stages 16 and 24 h. (A) Y70_16h *vs*. Y70_24h, (B) H5Y13_16h *vs*. H5Y13_24h. Each point represents a protein. (C) Y70_16h *vs*. Y70_24h, (D) H5Y13_16h *vs*. H5Y13_24h.

prominent differences in protein expression across 2 growth stages were observed in proteins effecting on monooxygenase activity, oxidoreductase activity, binding, membrane, and oxidation-reduction process (Figs. 6A and C). The main detected proteins involved in these pathways were: mitochondrial, acyl-coenzyme A oxidase, respiratory growth induced protein, NADH dehydrogenase, NADH oxidase N-terminal domain-containing protein, cytochrome P450, nitric oxide dioxygenase, and squalene monooxygenase. In Y70, the most notable differentially expressed proteins between the 24- and 16-h time points were citrate synthase, mitochondrial enzyme, fumarate hydratase, peroxisomal, acyl-coenzyme A oxidase, ATPase inhibitor, and protease A inhibitor. These proteins were involved in pathways of oxidation-reduction process, tricarboxylic acid cycle (TCA cycle), energy derivation by oxidation of organic compounds, and oxidoreductase activity function.

The results of KEGG enrichment analysis (Figs. 6B and D, and S11-S12) showed that significantly affected pathways of H5Y13 were the peroxisome, fatty acid metabolism, fatty acid degradation, carbon metabolism, and glyoxylate and dicarboxylate metabolism. The differentially expressed proteins involved in these pathways included citrate synthase, mitochondrial enzyme, acetyl-CoA carboxylase, malate dehydrogenase, and formate dehydrogenase. In Y70, significantly affected pathways were TCA cycle, carbon metabolism, biosynthesis of secondary metabolites, biosynthesis of antibiotics, and glyoxylate and dicarboxylate metabolism. The differentially expressed proteins involved in these pathways include citrate synthase, mitochondrial enzyme, fumarate hydratase, alcohol dehydrogenase, acyl-coenzyme A oxidase, and peroxisomal. The analysis of COG function classification of 2 strains revealed that the proteins related to structure and biogenesis stay dominate (Figs. S13 and S14).

The identification of differential expression of proteins during 2 distinct growth stages in Y70 and H5Y13 could contribute to elucidate the metabolic mechanisms of global protein synthesis. For example, acetyl-CoA carboxylase is a key enzyme in the introduction of acetyl-CoA into the biosynthetic pathway to numerous metabolites in brewing yeast and it enhances cell growth by upregulating the supply of cytoplasmic acetyl-CoA[29-30]. Peroxisomal enzymes play a crucial role in intracellular peroxide clearance. Upregulation of

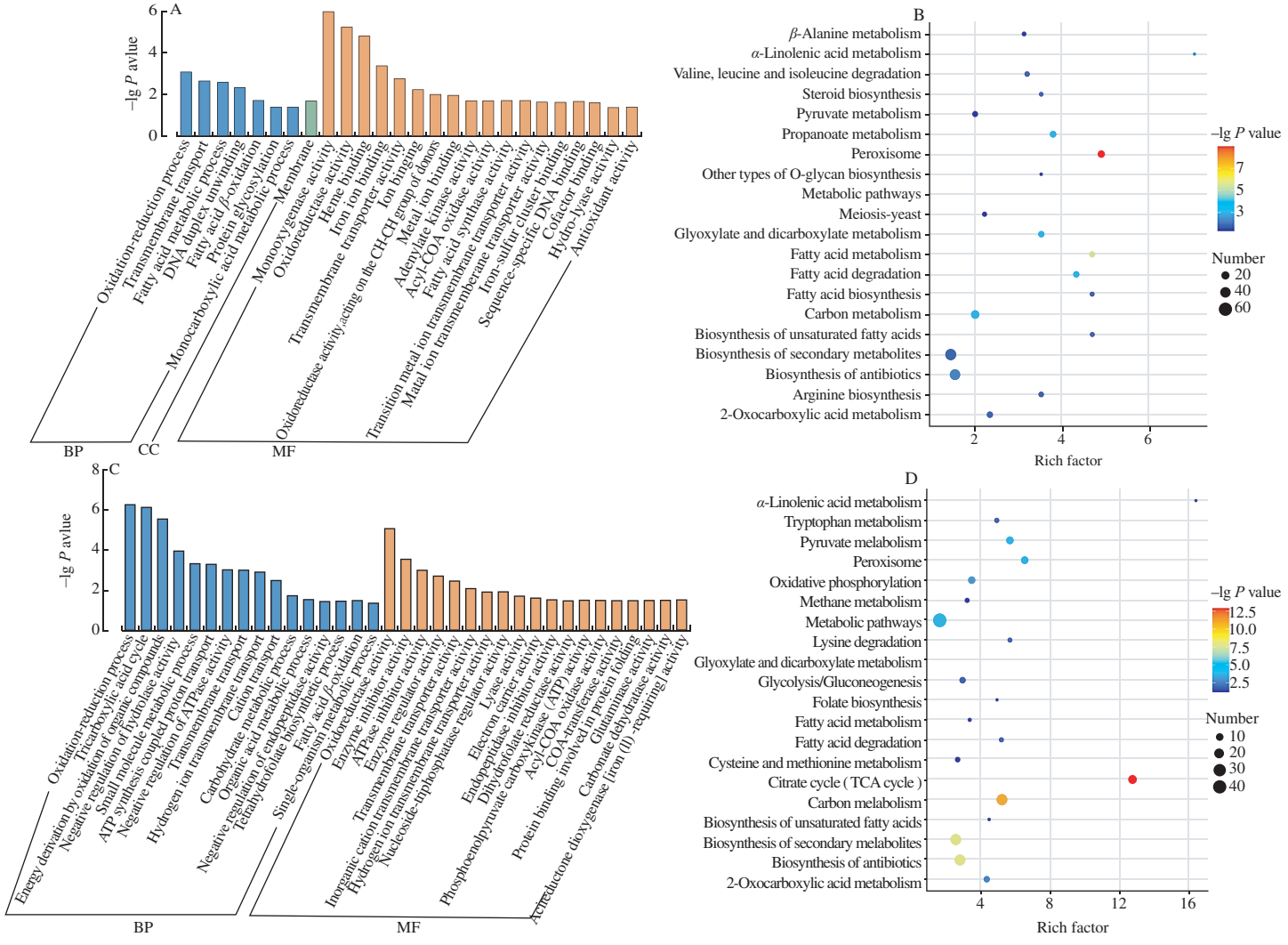

Fig. 6 Proteins of H5Y13 and Y70 were analyzed for GO and KEGG enrichment at the 16- and 24-h growth stages. (A) Enriched GO (H5Y13_24h *vs*. H5Y13_16h) and (C) enriched GO (Y70_24h *vs*. Y70_16h) was top 30 terms in GO enrichment analysis. (B) H5Y13_24h *vs*. H5Y13_16h and (D) Y70_24h *vs*. Y70_16h show KEGG enriched top 20 pathways. BP, biology process; CC, cellular component; MF, molecular function.

peroxisomal catalase A enhances the capacity for peroxide clearance, potentially impacting the energy and substrate supply for protein synthesis. Citrate synthase, as a key enzyme in the TCA, can elevate the cellular citrate content when its activity increases, thereby influencing carbon metabolic pathways. This, in turn, may affect yeast cell glucose utilization, organic acid production, and other metabolic products, ultimately regulating cell growth and global protein synthesis. Furthermore, certain intermediates participate in protein synthesis pathways within the cell's chromatin. Therefore, upregulating citrate synthase may potentially increase cellular organic acid and metabolic product abundance, consequently promoting the synthesis of specific amino acids and enhancing the rate of global protein synthesis[31]. In addition, the proteins involved in membrane play a crucial role in regulating various cellular processes, and is essential for ensuring proper cell growth and division, as well as for protecting mitochondrial DNA[32] and maintaining the permeability and fluidity of cell membranes. Moreover, squalene monooxygenase is an enzyme involved in cholesterol synthesis. When squalene monooxygenase is downregulated, cholesterol synthesis can be affected, potentially leading to lower cholesterol levels, which can change the structure of the cell membrane, thus affecting the localization, transport and intracellular signal transduction of membrane proteins[33].

Moreover, in these 2 yeast strains with different growth stages (16 and 24 h), the oxidative metabolism pathways, including the TCA cycle, oxidative phosphorylation, and the pentose phosphate pathway, have a significant impact on protein expression. These pathways are vital for providing the energy and building blocks necessary for protein synthesis. For instance, the TCA cycle generates NADH and $FADH_2$, which are used in the oxidative phosphorylation pathway to produce ATP, the primary energy source for cellular processes, including protein expression. Additionally, the pentose phosphate pathway generates ribose for nucleotide synthesis and NADPH for anabolic reactions, both of which are essential for protein production. Manipulating these oxidative metabolism pathways could be employed to engineer strains with enhanced protein production capabilities. For example, overexpressing key enzymes involved in the TCA cycle or oxidative phosphorylation could potentially increase the availability of energy and building blocks for protein synthesis, ultimately enhancing protein production. Similarly, modifying the flux through the pentose phosphate pathway could provide more precursor molecules for protein biosynthesis. Additionally, balancing the redox state within the cell, possibly through manipulation of NADH/NAD^+ and NADPH/$NADP^+$ ratios, could also augment the cellular capacity for protein production.

To sum up, the results of proteomic analysis indicated that at different growth stages, several proteins involved in the regulation of protein expression for both strains were significantly associated with the TCA, energy derivation through oxidation of organic compounds, and oxidoreductase activity function. Based on these findings, it can be inferred that the interplay between oxidative stress resistance and protein production plays a crucial role in the overall metabolic capabilities and cellular fitness of these 2 yeast strains. Yeasts that can effectively manage oxidative stress while maintaining robust protein production is likely to exhibit greater metabolic fitness, adaptability to changing environments, and potentially improved protein productivity in industrial applications. Therefore, understanding and optimizing this interplay may be essential for enhancing the fitness and protein-producing performance in yeast. Furthermore, these findings also provide potential molecular targets for constructing highly efficient yeast cell factories for protein production.

In addition, we simultaneously determined the essential amino acids (EAA) and protein efficiency ratio (PER) of the protein extract of Y70. We found that it is rich in essential amino acids, containing 9 EAAs (including threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine and tryptophan), with a total content as high as 43%. Its protein efficiency ratio is 2.51–0.03 (data not shown), proving that its crude protein reaches high quality. Therefore, it can be inferred that the selected strains not only have the potential for high protein production, but they also possess highly valuable nutritional properties of proteins and peptides, warranting further in-depth research.

4. Conclusion

In summary, this study has demonstrated that two yeast strains (*S. cerevisiae* strain Y70 and *C. parapsilosis* strain H5Y13), isolated from traditional Chinese Dong fermented pork (Nanx Wudl), possessed suitable technological characteristics for SCP production. Proteomics analysis suggested that some proteins might be rational targets for enhancing global protein synthesis ability, especially proteins related to TCA, energy derivation through oxidation of organic compounds, and oxidoreductase activity function, providing further insights for effective genetic engineering modifications in yeast.

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 herein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250356.

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