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Exploring the Fermentation Potential of *Kluyveromyces marxianus* NS127 for Single-Cell Protein Production

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Abstract: *Kluyveromyces marxianus* is a food-grade yeast known for its diverse beneficial traits, making it an attractive candidate for both food and biotechnology applications. This study explores the potential of *Kluyveromyces marxianus* as a promising alternative protein source for single-cell protein (SCP) production. Various *Kluyveromyces* strains were isolated and screened from traditional fermented dairy products, with *Kluyveromyces marxianus* NS127 identified as the most promising strain due to its superior growth characteristics, high SCP yield, and environmental tolerance. Notably, *Kluyveromyces marxianus* NS127 demonstrated significant substrate conversion capacity with a biomass yield of 0.63 g biomass/g molasses, achieving a dry biomass concentration of 66.64 g/L and a protein yield of 28.37 g/L. The protein extracted from the dry biomass exhibited excellent solubility (62.55%) and emulsification properties (13.15 m^2/g) under neutral conditions, alongside high foaming stability (93.70–99.20%) across a broad pH range (3–11). These results underscore the potential of *Kluyveromyces marxianus* NS127 as a viable alternative protein source and provide a solid theoretical foundation for its industrial application.

Keywords: *Kluyveromyces marxianus*; single-cell protein; production efficiency; environmental tolerance; protein characterization

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1. Introduction

In the coming years, the global population is projected to reach 9.3 billion, driving a 40% increase in the demand for protein by 2050 [1]. Traditional methods such as agriculture, animal husbandry, and aquaculture are increasingly constrained by land degradation, water scarcity, and climate change, making it difficult to meet this growing demand [2,3]. In this context, single-cell protein (SCP), derived from microorganisms such as bacteria [4], fungi [5], yeasts [6], and microalgae [7], has emerged as a promising solution. With its minimal land requirements, ability to utilize industrial by-products, and independence from climate or seasonal changes, SCP production offers a sustainable nutritional supplement for both humans and animals [8,9].

Yeasts are ideal for the production of SCP due to their high protein content, small cell size, ease of cultivation, and cost-efficiency [10,11]. Several yeast species, including *Saccharomyces* [12], *Candida* [13], *Pichia* [14], and *Kluyveromyces* [15], have shown potential

in the production of SCP. Among them, *Kluyveromyces marxianus* has garnered significant research interest. This yeast is widely present in dairy products and is classified as Generally Recognized As Safe (GRAS) [16]. It has been widely applied in the food industry, including the production of flavor compounds [17], enzymes [18], and emulsifiers [19]. Moreover, *Kluyveromyces marxianus* can efficiently utilize low-cost substrates such as molasses [20], cheese whey [21], potato pulp [22], and other agro-industrial waste materials [23]. It is also known for its rapid growth rate and resilience to environmental stress [24]. In addition, *Kluyveromyces marxianus* is a Crabtree-negative yeast, which prevents ethanol accumulation during aerobic fermentation [16]. These characteristics make *Kluyveromyces marxianus* an attractive candidate for industrial applications.

Previous studies have demonstrated the ability of *Kluyveromyces marxianus* strains to produce SCP [21,25,26], for instance, *Kluyveromyces marxianus* EXF-5288 showed that it could produce SCP with a high protein content and a balanced amino acid profile when cultured in deproteinized cheese whey [15]. However, the full potential of *Kluyveromyces marxianus* for SCP production remains underexplored, particularly in the context of selecting optimal industrial strains and fermentation optimization to enhance protein yield and quality. Furthermore, SCPs have been shown to possess superior functional properties, such as excellent emulsification, foaming, and heat stability [27,28]. These properties make SCP an attractive novel protein source with extensive application potential.

This study aims to explore the potential use of *Kluyveromyces marxianus* in SCP production. The strains were initially screened based on their growth rate, protein production capacity, and environmental tolerance. Single-factor and orthogonal experiments were conducted to optimize the fermentation conditions for SCP production using *Kluyveromyces marxianus* NS127. Subsequently, fed-batch cultivation was performed in a bioreactor. Finally, the solubility, emulsifying properties, and foaming properties of the extracted SCP were characterized. These findings contribute to the further development of *Kluyveromyces marxianus* NS127 as a viable alternative protein source.

2. Materials and Methods

2.1. Sampling

A total of 30 traditional fermented dairy products were collected using sterile samplers from local farms in the Ulanqab pasturing area, Inner Mongolia Autonomous Region; these products included 10 yogurt samples, 10 milk curd samples, and 10 Mongolian milk chew samples. All samples were transported in a refrigerated container and maintained at 4 °C until further processing.

2.2. Isolation and Identification of Kluyveromyces

The samples were homogenized using sterile homogenization bags and then serially diluted in 0.1% (*w*/*v*) peptone water (10^{-1} to 10^{-5}) [29]. Each dilution was plated with a 100 µL aliquot onto *Kluyveromyces marxianus* selection medium, which contained 5 g/L yeast extract, 3 g/L peptone, 3 g/L malt extract, 10 g/L glucose, 20 g/L agar, 200 mg/L chloramphenicol, 250 mg/L sodium propionate, and 35 mg/L X-Gal. The plates were subsequently incubated at 30 °C for 72 h [30,31]. Colonies exhibiting distinct blue phenotypes were selected and sub-cultured on yeast extract–malt extract agar medium, which contained 5 g/L peptone, 3 g/L malt extract, 10 g/L glucose, 3 g/L yeast extract, and 20 g/L agar; this was performed to isolate single yeast colonies.

The colony and cell morphologies of the purified yeast strains were observed using an optical microscope. DNA was extracted using a fungal genomic DNA extraction kit (D2300 Solarbio, Beijing, China). The ITS region was then amplified with universal primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [32].

Sequencing was performed by Beijing Ruiboxing Biotechnology Co., Ltd. (Beijing, China). The sequence data were analyzed and compared, using the NCBI database (https://www.ncbi.nlm.nih.gov/) to identify the species, accessed on 27 August 2024. A phylogenetic tree was then constructed using MEGA 11 software (MEGA Limited, Auckland, New Zealand) [33].

2.3. Screening of Kluyveromyces marxianus Strains with High SCP Production

2.3.1. Specific Growth Rate

The specific growth rate (μ) was determined using OD_{600} measurements that were obtained during the exponential growth phase. Then, 190 µL of yeast extract peptone dextrose (YPD) medium and ammonium chloride-based (ACB) medium (containing 20 g/L glucose, 1 g/L KH_2PO_4, 3.8 g/L NH_4Cl, 1 g/L $MgSO_4 \cdot 7H_2O$, 75 mg/L $CaCl_2 \cdot 2H_2O$, 15 mg/L EDTA-2Na, 4.5 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $MnCl_2 \cdot 7H_2O$, 0.3 mg/L $CuSO_4 \cdot 5H_2O$, 0.4 mg/L $Na_2MoO_4 \cdot 2H_2O$, 3 mg/L $FeSO_4 \cdot 7H_2O$, 0.1 mg/L KI, 0.1 mg/L biotin, 1 mg/L calcium pantothenate, 1 mg/L niacin) were dispensed into each well of a 96-well plate. Then, 10 µL of each *Kluyveromyces marxianus* strain, previously activated and standardized to 1×10^8 CFU/mL, was inoculated into the wells and cultured (30 °C, 150 rpm). The optical density at 600 nm was recorded every 4 h using a microplate reader (MK3, Thermo Fisher Scientific, Waltham, MA, USA), with blank media serving as the control. The μ was calculated using the following formula:

$$\mu_{max} = (\ln(OD_1) - \ln(OD_2))/(t_1 - t_2) \quad (1)$$

where OD_1 and OD_2 are the optical densities at 600 nm at times t_1 and t_2, respectively [34].

2.3.2. Screening High Protein Yield Strains

Kluyveromyces marxianus strains were inoculated into YPD medium and incubated at 30 °C via shaking at 200 rpm for 15 h, performed to establish a seed culture. The seed culture was then standardized to 1×10^8 CFU/mL, inoculated into 50 mL ACB medium (5% (v/v)). The culture was fermented at 30 °C via shaking at 200 rpm for 24 h, after which the dry biomass was collected to evaluate the production of SCP.

2.3.3. Environmental Tolerance

The environmental tolerance of *Kluyveromyces marxianus* strains under different conditions was evaluated based on OD_{600} measurements. Seed cultures were prepared as described above and inoculated at 5% (v/v) into a 96-well plate containing ACB medium with varying concentrations of ethanol (2%, 4%, 6%, 8%, and 10% (v/v)) and glucose (100, 200, 300, 400, and 500 g/L). The pH tolerance was evaluated by adjusting the pH of the ACB medium to 1.5, 2.5, 3.5, 4.5, and 5.5. All cultures were incubated at 30 °C with shaking at 150 rpm for 24 h. Temperature tolerance was evaluated by incubating the cultures at 25, 30, 35, 40, and 45 °C [35]. The optical density at 600 nm was measured using a microplate reader to determine the ethanol, glucose, pH, and temperature tolerance, with blank medium serving as the control [36].

2.4. Determination of Yeast Dry Biomass and Protein Content

The dry biomass (g/L) was determined according to the method described by Liu et al. [37], with some modifications. Yeast cells were collected by centrifuging the fermentation broth at 5000 rpm for 10 min. The biomass was then washed with distilled water and centrifuged again under the same conditions. The residue was dried at 60 °C until a constant weight was obtained.

The protein content (%, *w*/*w*) was measured using a protein analyzer (DN3000, Beijing Nord-Tech Instrument Co., Ltd., Beijing, China) according to the Dumas combustion method, with a nitrogen-to-protein conversion factor of 6.25. The protein yield (g/L) was calculated by multiplying the biomass by its protein content.

2.5. Effect of Different Medium Components on the SCP Production

In total, 20 g/L of maltose, corn starch, lactose, glucose, sucrose, or molasses was added to ACB medium (no carbon source) to evaluate the carbon effect on SCP production. The optimal carbon source identified was tested at concentrations of 10, 20, 30, 40, and 50 g/L. In addition to this, the effects of varying concentrations of KH_2PO_4 (1, 2, 3, 4, 5 g/L), NH_4Cl (3.8, 5.7, 7.6, 9.5, 11.4 g/L), and corn steep liquor (3, 6, 9, 12, 15 g/L) on the production of SCP using NS127 were also evaluated. The inoculums (5% (*v*/*v*)) were cultured at 30 °C with shaking at 200 rpm for 24 h without pH adjustment. Furthermore, a L_9 (3^4) orthogonal array design was used to optimize the production of SCP using NS127 (Table S1).

2.6. Fed-Batch Fermentation Process for SCP Production

The initial medium (M1) consisted of molasses (25 g/L), KH_2PO_4 (3 g/L), NH_4Cl (2.85 g/L), corn steep liquor (15 g/L), $MgSO_4{\cdot}7H_2O$ (75 mg/L), $CaCl_2{\cdot}2H_2O$ (15 mg/L), EDTA-2Na (4.5 mg/L), $ZnSO_4{\cdot}7H_2O$ (1 mg/L), $MnCl_2{\cdot}7H_2O$ (0.3 mg/L), $CuSO4{\cdot}5H_2O$ (0.4 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (3 mg/L), $FeSO_4{\cdot}7H_2O$ (0.1 mg/L), KI (0.1 mg/L), biotin (0.1 mg/L), calcium pantothenate (1 mg/L), and niacin (1 mg/L). The feeding medium (M2) was prepared as a 10-fold concentrated version of M1, with a total volume of 1.5 L. The experiments were conducted in a 5 L bioreactor with an initial working volume of 2 L, and the seed culture was set at 10% (*v*/*v*). During the fed-batch process, the temperature was maintained at 30 °C, with stirring speeds ranging from 400 to 800 rpm, adjusted based on the dissolved oxygen levels automatically. The total sugar concentration in the bioreactor was maintained between 5 and 25 g/L, and the dissolved oxygen (DO) levels were controlled within the range of 15 to 30%. Total sugar content was monitored using the phenol–sulfuric acid method. The airflow rate was maintained at 1 L of air per liter of culture per minute, with the pH naturally controlled (4.5–5.5).

2.7. Characterization of Kluyveromyces marxianus NS127 Protein Properties

2.7.1. Protein Extraction

The fermentation broth was sterilized by heating at 100 °C for 25 min. The cells were centrifuged at 4 °C and 5000× *g* for 10 min to collect the biomass, then washed twice with distilled water and freeze-dried to obtain dry biomass. The dry biomass was reconstituted into a 10% (*w*/*v*) dispersion with deionized water. Cell disruption was carried out using an ultrasonic cell disruptor (SCIENTZ-650E, Scientz Biotechnology Co., Ltd., Ningbo, China) at 150 W for 20 min at 25 °C [38]. The disrupted suspension was then centrifuged at 4 °C for 10 min at 6000× *g* to separate the supernatant. For protein extraction, the pH was adjusted to 4.5 with 1 M HCl, followed by three gentle washes. The extracted protein was freeze-dried after the pH was neutralized.

2.7.2. Protein Solubility

The protein solubility was determined by dispersing the freeze-dried protein in deionized water at different pH levels (3–11). The protein suspension was stirred at 25 °C for 1 h, centrifuged at 4 °C for 15 min at 7000× *g*, and the supernatant was collected so that the protein concentration could be measured using the BCA method. The protein solubility was calculated as the ratio of soluble protein concentration to the total protein concentration.

2.7.3. Emulsification Capacity and Stability

In total, 5 milliliters of soybean oil were added to a beaker containing 15 mL of protein dispersion (10 mg/mL), with pH levels ranging from 3 to 11. The mixture was then homogenized at 12,000 rpm for 1 min. At 0 and 10 min, 50 µL aliquots of the emulsion were collected from the bottom of the beaker and dispersed in 5 mL of 0.1% (*w*/*v*) sodium dodecyl sulfate solution. The absorbance was then measured at 500 nm using a spectrophotometer. The emulsifying activity index (EAI) and emulsifying stability index (ESI) were calculated using the following formulas [27]:

$$\mathrm{EAI}\left(\mathrm{m}^2/\mathrm{g}\right) = 2 \times 2.303 \times A_0/C \times \varphi \times 1000 \times D \quad (2)$$

$$\mathrm{ESI}(\mathrm{min}) = A_0/A_0 - A_{10} \times \Delta t \quad (3)$$

where A_0 and A_{10} represent the absorbance of the emulsion at 500 nm at 0 min and 10 min, respectively, C is the protein concentration, D is the dilution factor, φ is the oil phase ratio (0.25), and Δt = 10 min.

2.7.4. Foaming Property

The foaming capacity (FC) and foam stability (FS) were assessed according to the method established by Ye et al. [39], with some modifications. In total, 15 milliliters of protein dispersion (10 mg/mL, pH 3–11) was placed in a 50 mL graduated cylinder and sheared at 13,600 rpm for 2 min. The total volume of the sample solution and the foam produced was recorded at 0 min (V_0) and 10 min (V_{10}). The FC and FS were calculated using the following formulas:

$$\mathrm{FC}(\%) = V_0/15 \times 100\% \quad (4)$$

$$\mathrm{FS}(\%) = V_{10}/V_0 \times 100\% \quad (5)$$

2.8. Statistical Analysis

The graphs were plotted using OriginPro 2024 software (OriginLab Corporation, Northampton, MA, USA). The data are presented as the mean ± standard deviation (SD) from three independent experiments, each performed in triplicate. Statistical analysis was carried out using SPSS 19.0, with one-way ANOVA followed by Tukey's post hoc test for significance ($p < 0.05$).

3. Results and Discussion

3.1. Isolation and Identification of Kluyveromyces Strains

A total of 167 microbial colonies were isolated from 30 samples of traditional fermented dairy products according to chromogenic reactions and the morphological characteristics. Among these, 72 strains were isolated from Mongolian milk chew, 61 strains were isolated from yogurt, and 34 strains were isolated from milk curd. ITS sequence analysis was performed to identify strains with identical sequences; these were then removed, and 11 different strains were identified, including 10 *Kluyveromyces marxianus* and 1 *Kluyveromyces lactis*. As shown in Figure 1a, the diversity of *Kluyveromyces* in Mongolian milk chew (11 strains) was significantly higher than that in yogurt (3 strains) and milk curd (2 strains). *Kluyveromyces lactis* was isolated exclusively from Mongolian milk chew. Mongolian milk chew is fermented under natural conditions with minimal human intervention, which may contribute to the higher microbial diversity observed in Mongolian milk chew compared to yogurt and milk curd. The observed differences in strain diversity may be related to the production process and physicochemical properties of the samples [40,41].

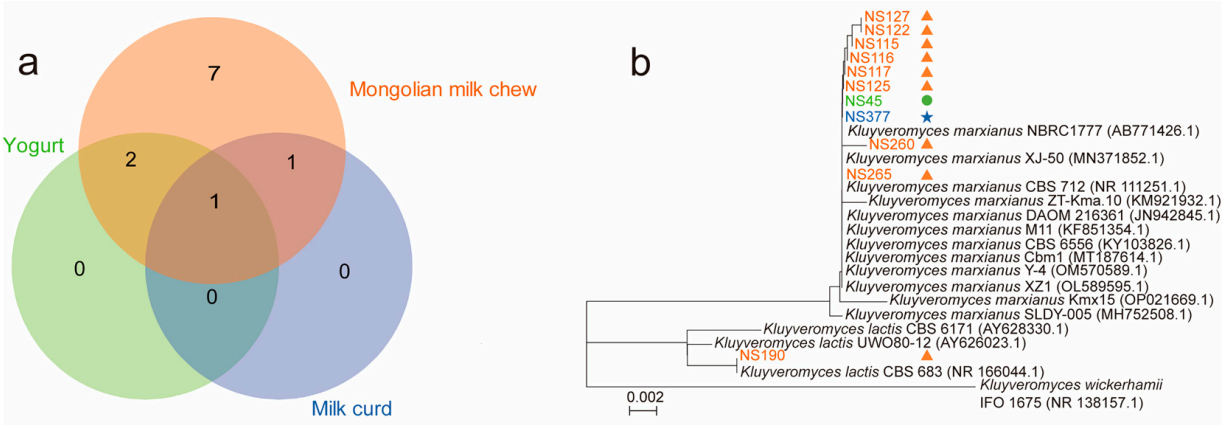

Figure 1. (**a**) Distribution of differentiated strains isolated from three traditional fermented dairy products; (**b**) phylogenetic tree of the selected strains. The symbols represent the sample isolation source (▲ from Mongolian milk chew, ● from yogurt, ★ from milk curd).

To investigate the evolutionary relationships among these strains, *Kluyveromyces wickerhamii* was selected as the outgroup to construct a phylogenetic tree (Figure 1b). The phylogenetic analysis revealed that 10 of the isolated strains clustered with the *Kluyveromyces marxianus* reference strains, displaying varying degrees of evolutionary divergence. One strain, identified as *Kluyveromyces lactis* NS190, clustered with the *Kluyveromyces lactis* CBS 683, confirming its classification. The high intraspecific diversity within *Kluyveromyces marxianus* strains has been reported to potentially impact their physiological characteristics [42,43]. Therefore, it is essential to further characterize the physiology of these *Kluyveromyces marxianus* strains, which could be utilized in SCP production.

3.2. Specific Growth Rates, SCP Production, Tolerance Assessment

The specific growth rates (μ) of ten *Kluyveromyces marxianus* strains were measured in both YPD medium and ammonium chloride basal (ACB) medium (Figure 2a). In YPD medium, the μ values ranged from 0.20 to 0.44 h^{-1}. The highest μ value was observed in NS45. In the ACB medium, the μ values were slightly lower, ranging from 0.16 to 0.43 h^{-1}, with NS127 reaching the highest μ value of 0.43 h^{-1}. In general, stains cultured in mineral media exhibit lower growth rates compared to those in nutrient media [44]. However, six strains (NS127, NS125, NS122, NS260, NS265, and NS377) showed higher growth rates in ACB medium than in YPD medium. This suggests that they have a higher utilization efficiency for inorganic nitrogen sources. The dry biomass values ranged from 2.97 to 4.74 g/L, and the protein content varied from 31.16% to 42.86% (w/w) (Figure 2b). Among these strains, NS265, NS127, and NS122 exhibited higher protein yields of 1.66 g/L, 1.64 g/L, and 1.58 g/L, respectively. These strains were selected for further tolerance analysis.

Three strains (NS127, NS122, and NS265) were assessed for their tolerance to ethanol, glucose, pH, and temperature. NS127 and NS265 exhibited tolerance to 10% (v/v) ethanol (Figure 2c), consistent with that of *Kluyveromyces marxianus* FIM1 after adaptive evolution [45]. In contrast, NS122 was more sensitive to ethanol, showing only minimal growth under the same conditions. Regarding glucose tolerance, all strains showed a decrease in growth as the concentration of glucose increased but maintained significant osmotic tolerance up to 400 g/L (Figure 2d). They tolerated a broad pH range from 2.5 to 5.5 (Figure 2e), with the lowest pH tolerance being comparable to results reported by Amrane and Prigent [46]. Regarding temperature tolerance, NS127 showed higher OD_{600} values across all temperatures than NS122 and NS265, indicating superior growth performance under these thermal conditions (Figure 2f). Overall, NS127 was chosen for further investi-

gation owing to its remarkable growth rate in inorganic nitrogen media, balanced ability to produce protein, and environmental tolerance.

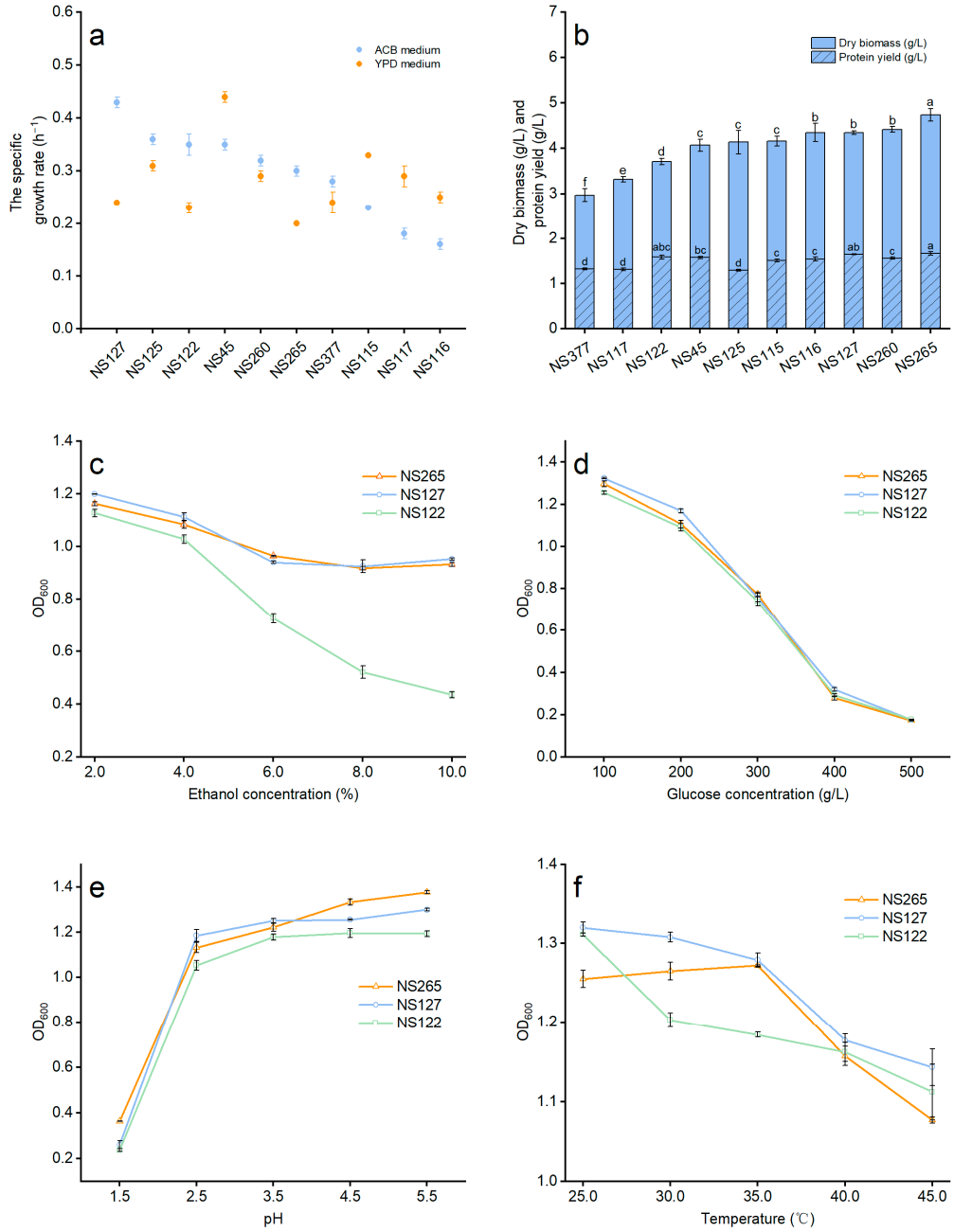

Figure 2. Growth characteristics and tolerance of selected *Kluyveromyces marxianus* strains: (**a**) Specific growth rates (μ) in different media; (**b**) dry biomass and protein yield in ACB medium; (**c**) ethanol tolerance; (**d**) glucose tolerance; (**e**) pH tolerance; (**f**) temperature tolerance. Different parameter superscripts indicate significant differences ($p < 0.05$).

3.3. Optimization of Carbon, Nitrogen, and Phosphorus Sources in the Culture Media

The effect of various carbon, nitrogen, and phosphorus sources on the dry biomass and protein yield of *Kluyveromyces marxianus* NS127 was evaluated. The dry biomass of NS127 ranged from 0.93 g/L to 4.93 g/L (Figure 3a). The protein yield ranged from 0.54 g/L to 2.35 g/L when using different carbon sources (maltose, corn starch, lactose, glucose, sucrose, and molasses). The protein content in the dry biomass across these media ranged from 37.50% to 58.06% (*w*/*w*). Among these, molasses was the preferable carbon source for the production of SCP, followed by sucrose and glucose. Molasses contains many

fermentable sugars, such as sucrose, glucose, and fructose, as well as valuable non-sugar organic compounds and inorganic salts [47]. The synergistic effects of these components may enhance both the growth and protein yield of NS127. It is noteworthy that dry biomass was significantly higher at a lower concentration of molasses (10–20 g/L), whereas the protein yield remained relatively stable (Figure 3b). These results suggest that regulating the total sugar concentration during fermentation could help enhance protein yield.

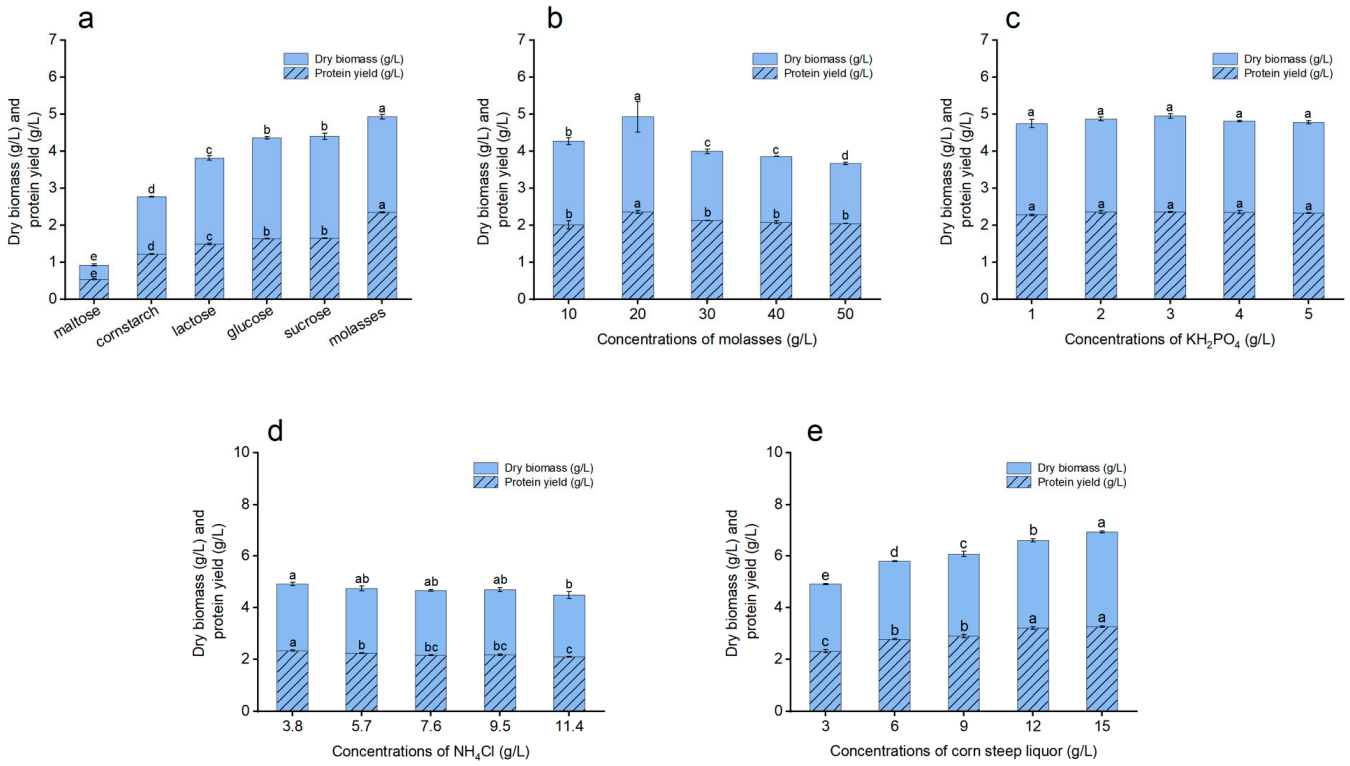

Figure 3. Effect of various components on the dry biomass and protein yield of *Kluyveromyces marxianus* NS127: (**a**) carbon sources; (**b**) molasses concentrations; (**c**) KH_2PO_4 concentrations; (**d**) NH_4Cl concentrations; (**e**) corn steep liquor concentrations. Different parameter superscripts indicate significant differences ($p < 0.05$).

The dry biomass and protein yield of NS127 did not vary significantly as the concentration of KH_2PO_4 increased (1–5 g/L) (Figure 3c). Wang et al. observed a reduction in the dry biomass of *Kluyveromyces marxianus* FXJ1 when the concentration of KH_2PO_4 exceeded 4 g/L [48]. This difference shows that NS127 could be more adaptable to changes in the concentration of KH_2PO_4, which may enhance the flexibility of the medium formulation.

With the increase in NH4Cl concentration, neither the dry biomass nor the protein yield was enhanced as expected (Figure 3d). Further adding corn steep liquor was tested to improve protein yield. The dry biomass reached 6.91 g/L, and the protein yield reached 3.26 g/L upon the addition of 15 g/L corn steep liquor (Figure 3e). This represented a 40% improvement in protein yield compared to the basal medium containing only inorganic nitrogen source. This suggests that the addition of NH4Cl along with corn steep liquor enhances protein yield, offering a more favorable balance between cost and production efficiency.

3.4. Optimization of Protein Yield Through Orthogonal Experiments

The composition of the media was optimized using an L_9 (3^4) orthogonal design to maximize the SCP yield produced by *Kluyveromyces marxianus* NS127. As shown in Table 1, factor A had the highest range (R) value of 0.61, followed by factors D (0.29), B (0.10), and

C (0.07). A higher range indicates a greater effect on protein yield. Hence, the factors influencing protein yield were ranked as follows: molasses > corn steep liquor > NH_4Cl > KH_2PO_4. Additionally, the analysis of variance showed that molasses had a significant effect on protein yield ($p < 0.05$) (Table S2). Based on the mean (K) values of the factors, the optimal combination was determined as $A_3B_1C_3D_3$. This combination corresponds to a media formulation containing molasses (25 g/L), NH_4Cl (2.85 g/L), KH_2PO_4 (3 g/L), corn steep liquor (15 g/L), and trace elements (designated as M1; see Section 2.6 for details).

Table 1. The results of the orthogonal experiment.

Test Number	Factor				Protein Yield (g/L)	Dry Biomass (g/L)
	A. Molasses (g/L)	B. NH_4Cl (g/L)	C. KH_2PO_4 (g/L)	D. Corn Steep Liquor (g/L)		
1	15	2.85	1	9	2.74 ± 0.04	6.04 ± 0.04
2	15	3.80	2	12	2.92 ± 0.01	6.35 ± 0.11
3	15	4.75	3	15	3.00 ± 0.10	6.50 ± 0.08
4	20	2.85	2	15	3.41 ± 0.06	7.24 ± 0.02
5	20	3.80	3	9	3.09 ± 0.01	6.65 ± 0.02
6	20	4.75	1	12	3.13 ± 0.12	6.59 ± 0.04
7	25	2.85	3	12	3.59 ± 0.00	7.11 ± 0.16
8	25	3.80	1	15	3.60 ± 0.09	7.09 ± 0.09
9	25	4.75	2	9	3.30 ± 0.01	6.74 ± 0.02
K_1	2.89	3.25	3.16	3.04		
K_2	3.21	3.20	3.21	3.21		
K_3	3.50	3.14	3.23	3.34		
R	0.61	0.10	0.07	0.29		

To validate the predicted results, fermentation was conducted using $A_3B_1C_3D_3$ formulation, resulting in a dry biomass of 7.30 g/L and a protein yield of 3.66 g/L. As expected, the protein yield was 2.23-fold higher than the initial medium (1.64 g/L) and exceeded all orthogonal experiment groups (2.74–3.60 g/L). These results demonstrated the reliability of the optimized fermentation medium.

3.5. Fed-Batch Fermentation for SCP Production

The potential of NS127 to produce SCP in medium M1 was evaluated using a fed-batch culture in a 5 L bioreactor. Based on the values of DO (45.41%) and sugar concentration (20.5 g/L), feeding was initiated after 8 h fermentation. During the feeding process, DO levels were kept between 15% and 30% (Figure 4a). Meanwhile, the residual sugar concentration was maintained between 5 and 20 g/L. The cumulative added sugar with a final concentration of 121.4 g/L. Consequently, following 30 h fermentation, the dry biomass achieved a concentration of 66.64 g/L, while the SCP yield reached 28.37 g/L (Figure 4c). The carbon source conversion rate was 0.63 g biomass/g molasses, representing a 2.17-fold increase compared to the shake flask stage. The protein content of dry biomass reached nearly 59% during the initial 12 h but gradually decreased to 42.60% as the fermentation progressed. This decrease may be attributed to factors such as agitation, DO levels, or shear stress, all of which can affect the physiology and metabolism of cells [15]. Yadav et al. [21] reported the production of protein using cheese whey in a 10 L bioreactor, achieving a protein content of 42.0%; however, the biomass yield was only 0.19 g biomass/g lactose. Koukoumaki et al. [15] observed a final protein concentration of 37% and a biomass yield of 0.06 g biomass/g lactose when using *Kluyveromyces marxianus* EXF-5288. In summary,

NS127 can produce a high yield of SCP and a competitive yield of biomass in an optimized synthetic medium, indicating its strong potential for industrial applications.

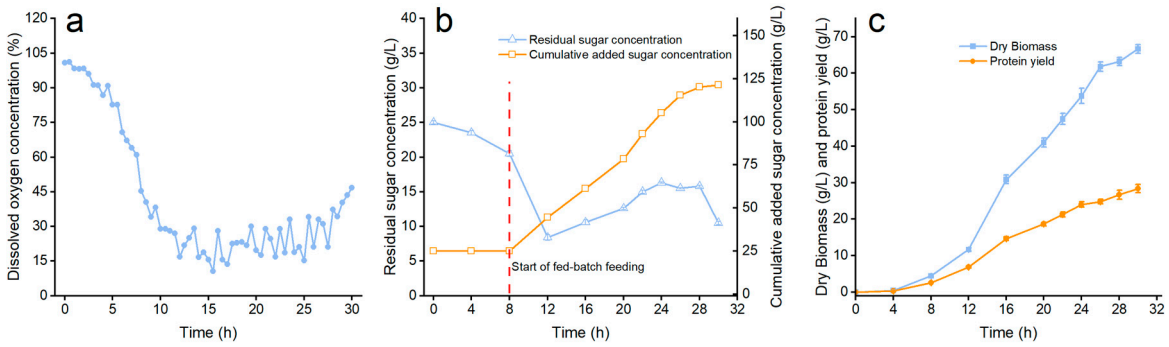

Figure 4. Process conditions of *Kluyveromyces marxianus* NS127 during the fed-batch fermentation in a 5 L bioreactor: (**a**) dissolved oxygen (DO); (**b**) residual sugar concentration and cumulative added sugar concentration in the medium; (**c**) dry biomass and protein yield.

3.6. Protein Solubility, Emulsifying and Foaming Properties

Protein solubility is a critical functional property in food applications, influencing emulsification, foaming, and other functionalities [49]. The protein content of NS127 SCP increased to 81.15% after cell disruption, protein extraction, and purification. Figure 5a presents the protein solubility curve at different pH values. Under acidic conditions, the protein exhibited low solubility, with the lowest solubility (1.25%) observed near its isoelectric point (pH 4.5). As the pH increases above 5.0, the solubility of protein increases significantly, reaching a maximum of 70.20% at pH 11. Notably, the protein solubility at pH 7.0 (62.55%) was higher than freeze-dried soy protein isolate (40.8%) [50], indicating that NS127 protein could be a viable alternative to soy protein isolate in the formulation of food products.

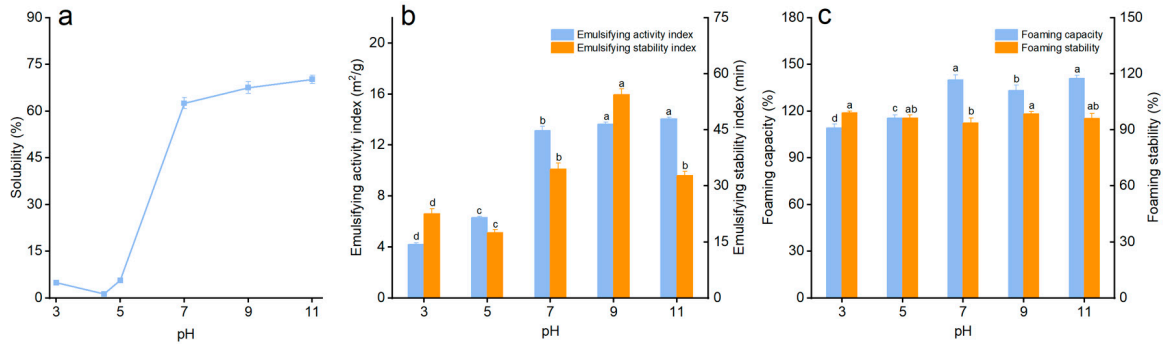

Figure 5. Functional properties of *Kluyveromyces marxianus* NS127 protein under different pH conditions: (**a**) protein solubility; (**b**) emulsification activity index (EAI) and emulsification stability index (ESI); (**c**) foaming capacity (FC) and foaming stability (FS). Different parameter superscripts indicate significant differences ($p < 0.05$).

Emulsifying ability refers to a protein's capacity to reduce interfacial tension and promote the formation of stable oil-water interfaces [51]. As shown in Figure 5b, the trend observed in the protein's emulsification activity index (EAI) at different pH levels closely aligns with its solubility profile. NS127 protein possesses significantly ($p < 0.05$) higher EAI under pH 9 and 11 than others. The emulsification stability index (ESI) of the protein differed slightly from its EAI, reaching its highest value at pH 9. This could be explained by the impact of surface hydrophobicity on the ESI once the solubility surpasses a certain threshold [52]. In particular, the emulsifying capacity of the protein at pH 7.0 (13.15 m^2/g) and pH 9.0 (13.65 m^2/g) exceeds that of eight traditional Chinese bean proteins

(9.10–10.33 m^2/g) [53]. Therefore, this protein could be an alternative to plant proteins for emulsifying applications.

The foaming capacity (FC) and foaming stability (FS) of the NS127 protein were evaluated under different pH conditions (Figure 5c). The lowest FC was observed at pH 3.0 (109.3%), while the highest values were recorded at pH 7.0 (140%) and pH 11.0 (141.1%). According to Garcia-Vaquero et al., the improved foaming capacity at higher pH levels can be attributed to an increase in the protein's net charge, which weakens hydrophobic interactions [54]. This reduction in hydrophobic interactions enhances the protein flexibility, enabling it to rapidly migrate to the air-water interface and facilitate foam formation. The FS of the protein remained high across all pH conditions (93.7–99.2%), likely due to its structural characteristics, which enable the formation of a strong network at the air-water interface.

4. Conclusions

Kluyveromyces marxianus NS127 was isolated and identified from traditional fermented dairy products, exhibiting a high specific growth rate and excellent single-cell protein production capacity. Additionally, it demonstrated remarkable environmental tolerance, including resistance to ethanol, glucose, pH variations, and temperature fluctuations. Fed-batch cultivation using molasses and corn steep as primary carbon and nitrogen sources resulted in a high carbon source conversion efficiency of 0.63 g biomass/g molasses and a high protein yield of 28.37 g/L. The extracted protein exhibited superior functional properties, underscoring its potential advantages for food processing applications. This study highlights the significant potential of *Kluyveromyces marxianus* NS127 as an alternative protein source. Further optimization of the fermentation process, large-scale cultivation, and cost-effectiveness evaluations will be crucial for its broader application in the food industry.

5. Patents

Lichao Dong, Yanyan Wu and Dingrong Kang are the inventors of the patent: A High-Density Cultivable *Kluyveromyces marxianus* Strain and Its Application in Single-Cell Protein Production. Patent No.: ZL 2024 1 0576609.7.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11020070/s1, Table S1: The factor level of orthogonal experiment; Table S2: Analysis of variance of orthogonal experiment results.

Author Contributions: Conceptualization, D.K.; methodology, L.D., M.L. and D.K.; software, L.D.; validation, Y.W. and W.Z.; formal analysis, Y.W.; investigation, L.D., Y.W. and J.C.; resources, D.K.; writing—original draft, L.D.; writing—review and editing, C.Z., R.L.-A., W.Z. and D.K.; visualization, L.D.; supervision, W.Z. and D.K.; project administration, D.K.; funding acquisition, D.K. All authors have read and agreed to the published version of the manuscript.

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and NS260 (PQ269320.1). The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: Authors Lichao Dong, Yanyan Wu, Mingxia Li, Jialu Cao and Dingrong Kang are employed by the DeePro Technology (Beijing) Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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