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Optimization of fermentation parameters for enhanced and low-cost production of single-cell protein from rice polishing using *Rhizopus oligosporus*

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Abstract

A growing concern over food shortages caused by the expanding world population has led to the use of unconventional substitutes for food sources, such as single-cell protein (SCP) produced from microbes using inexpensive feedstock and wastes. The fungal strain *Rhizopus oligosporus* can consume different substrates, and thus has been used for the production of several products for human and animal consumption. There are very few reports on using rice polishing for SCP production from microbes; however, there is no information regarding the use of *R. oligosporus*. This study aimed to optimize the process parameters to investigate the possibility of enhanced bioconversion of rice polishing into SCP using *R. oligosporus* in comparison with the control medium (glucose). Proximate composition of rice polishing as a potential substrate was estimated, and the effects of process variables on biomass and protein content were elucidated. The optimum conditions including substrate concentration 8%, temperature 30°C, pH 5.5, inoculum size 5%, fermentation period 96 h, and optimum amount of supplements in the medium were found to give a considerable biomass (19.5 ± 1.93 g/L) and protein content (49.5 ± 0.9%), which are comparable with the control medium. The optimum conditions were verified and statistically analyzed through confirmatory experiments in triplicate.

Keywords: agro-industrial wastes; biomass; microbial-derived protein; optimum variables; protein content

Introduction

Significant food shortages, particularly in developing countries, are one of the challenges the modern world is currently facing, and they also have an impact on human health. In that instance, the major issue for humanity is the shortage of protein as the population grows (Anupama and Ravindra, 2000). Therefore, in 1996 researchers started focusing on developing microorganisms such as bacteria, fungi, yeasts, and algae that are known for their rapid growth on waste and inexpensive materials such as industrial and agricultural residues to produce single-cell protein (SCP) as a new low-cost alternative of food sources (Nangul and Bhatia, 2013). The term "single-cell protein" was first used in 1960 and seems appropriate because most microbes grow as single or filamentous entities. It is also named as bioprotein, biomass, or microbial protein and is used as a supplement or alternative to protein-rich food sources, particularly in animal feeds or for human consumption (Kumari *et al*., 2018). Single-cell organisms likely offer the best possibility of developing distinct independence from agricultural-based food sources. The protein derived from microorganisms is inexpensive, with high nutritional value based on the amino acid content and may compete well with other protein sources. The expense of feeding goes up in case of balancing with high-quality protein. Therefore, it is essential to generate affordable protein biomass that is relatively comparable to animal protein (Singh *et al*., 1991). A number of fungal species are being used to produce SCPs. Fungi are known for their unique chemical composition and amino acid profile, and therefore are preferred over different protein-rich food sources (Ravinder *et al*., 2003). When grown primarily for the synthesis of SCP, fungi contain 30–50% protein. Additionally, their amino acid profile is compliant with FAO standards (Sharif *et al*., 2021). In addition to protein, SCP from fungi may also contain various other nutrients that mainly include vitamins, mostly from the vitamin B-complex (Nyyssölä *et al*., 2022).

Rhizopus oligosporus, which is not found in nature, is regarded as a domesticated strain of *Rhizopus microsporus*. It is not like other strains of *R. microsporus* in that it does not produce mycotoxins, rhizoxins, and rhizonins as secondary metabolites, and despite being domesticated, *R. oligosporus* still retains its exotic appearance due to the highly distinctive morphology associated with its large and irregular spores. Moreover, *R. oligosporus* grows faster than *R. microsporus* in terms of biomass while growing the same way in terms of radial growth at the temperature range of 30–40°C. Being a heterotroph, *R. oligosporus* can use a variety of food sources, including both organic and inorganic nitrogen sources, to best promote its growth (Jennessen *et al*., 2005). Most people are familiar with *R. oligosporus* from its use in the tempeh production, a staple dish in Southeast Asia. *R. oligosporus* is a key factor in the fermentation of soybeans into tempeh, an alternative to meat that is gaining popularity across the globe (Dwiatmaka *et al*., 2021). In addition, *R. oligosporus* also plays a key role in producing many industrial enzymes and in waste treatment processes (Jennessen *et al*., 2005).

Different industrial and agricultural wastes are available in greater quantity and at comparably less expense than other sources (Shahzadi *et al.,* 2024; Arifeen *et al.,* 2024). Additionally, 20–30% of the entire production costs are covered by these wastes. However, before being used for human consumption, solid agricultural wastes like cellulose must undergo specific, expensive processing (Aziz *et al.,* 2024). Moreover, industrial wastes have a high biological oxygen requirement, and if disposed of carelessly, they might pollute the environment (Dong *et al.,* 2025; Yang *et al.,* 2025). Therefore, by employing industrial and agricultural wastes, the biological oxygen demand for SCP production can be reduced by over 80% and it also lowers the cost of treatment needed for their disposal (Kam *et al.*, 2012). In Pakistan, the food industry is struggling to acquire high-quality ingredients from both plant and animal sources due to the high demand for an increasing population (Usman *et al.,* 2024; Hassan *et al.,* 2024). Moreover, animal protein is mainly provided by the poultry industry in the form of meat and eggs. One possible nonconventional alternative can be the agro-industrial wastes and residues that can be fermented to produce SCP as an additional protein source for livestock, poultry, and even humans (Abdullahi *et al*., 2021). The total amount of agro-industrial wastes that accumulate in this country is up to 50 million annually, including 1.7 million tons of rice polishing (Ibrahim Rajoka *et al*., 2004). Rice fulfills the 60–70% of calories' demand in more than 2 million people in Asia alone, making it one of the most important and significant crops in the world (Zheng *et al*., 2005). Rice polishing is a by-product of rice milling and is the most economical source for energy and protein in poultry feeding. Being a good source of protein, vitamins, minerals, and energy, rice polishing has great potential as an important ingredient in poultry feed (Das *et al*., 2008). It can be transferred into fermentable sugars through appropriate hydrolysis and can be utilized as a cultural substrate to grow microorganisms.

As discussed above, *R. oligosporus* is one of the best suitable stains, widely used in the food industry; therefore, it is being further explored for SCP production using different agricultural and industrial wastes (Mahat and MacRae, 1992; Mahnaaz et al., 2009; Yunus *et al*., 2015; Yousufi, 2012). Rice polishing is a key component in poultry feed due to it being a major source of protein, vitamins, minerals, and energy feed as mentioned above (Das *et al*., 2008). However, it is relatively a newly introduced and less reported agricultural waste to be used as substrate for SCP production by some microbial strains (Ahmed *et al*., 2017; Ibrahim Rajoka *et al*., 2004; Rajoka *et al*., 2006), and there is no such information reported regarding *R. oligosporus* except one report where this fungus was used as coculture with *Candida utilis* for nutritional upgradation of feed ingredients through SCP production by agricultural wastes, including rice polishing (Nadeem *et al*., 2016). The selection of the best stain and substrate, as well as suitable optimized process conditions, facilitates the improved production of protein biomass and quality. To design an effective fermentation process, it is crucial to optimize fermentation conditions and media composition in addition to developing superior strains through mutation (Singh *et al.*, 1991). Therefore, the current study aimed to determine the effects of culture conditions, such as temperature, pH, fermentation period, and substrate concentration, on the production of biomass and SCP by *R. oligosporus* using rice polishing medium in comparison with the control medium (glucose). In addition, the proximate analysis of substrate was also a part of this study to identify the potential of rice polishing as an effective substrate for improved protein yield with reduced production cost. The findings of this study could provide new opportunities for a variety of industrial uses of microbial protein and help meet the current global demands for protein.

Materials and Methods

Collection and processing of substrate

A sufficient amount of rice polishing as substrate was bought from the local market in Lahore, Pakistan. The substrate was passed through a 40-mesh sieve to remove different impurities and stored in transparent zip-locked polythene bags until further use.

Estimation of the proximate composition of rice polishing

The proximate analysis of rice polishing was determined using official methods of analysis established by the Association of Official Analytical Chemists (AOAC, 2005).

First, the moisture content of the substrate was determined by putting weighed rice polishing in a dry heating oven at 60°C for 24 h until constant weight was attained. Then, the per cent moisture content (%MC) was estimated. Further, the dry weight of 100 g of rice polishing was measured in terms of yield percentage. For estimation of crude protein (CP) content, the per cent nitrogen (%N) of dry biomass estimated by the Kjeldahl method was multiplied with factor 6.25 to calculate crude protein (%CP) following AOAC Method No. 978.04. Crude fat (FC) content was determined by the extraction of rice polishing in hexane using a Soxhlet apparatus at 60°C for 6 h, following AOAC Method No. 930.09, and the per cent FC in air-dried extract was estimated.

The fat-free material was then used to determine crude fiber (CF) by the gravimetric method using sequential extractions of materials other than fiber in acid and alkali. The weight of fiber residue was then adjusted for ash content after combustion. The ash content was determined by placing a weighed substrate at 550°C in a muffle furnace for 6 h. Then, the per cent CF and ash content (AC) were calculated (AOAC Method No. 930.05). The total carbohydrate content, also called as nitrogen-free extract (NFE), was determined by calculating the per cent remaining after all the other components have been measured (Traughber *et al.*, 2021). The energy content of the rice polishing was determined using the "Atwater factor" (Sánchez-Peña *et al.*, 2017). All experiments were carried out in three replicates, and the results, except for moisture content, were presented as dry weight.

Collection of fungal strains and preparation of inoculum

For the fermentation of rice polishing to produce SCP, the fungal strain, *R. oligosporus* (Acc. No: FCBP-SF-1056), was obtained from the Fungal Culture Bank of Pakistan (FCBP), Department of Plant Pathology, Faculty of Agricultural Sciences (FAS), University of the Punjab, Lahore, Pakistan. The fungus was maintained on potato dextrose agar (PDA) plates (pH = 5.5) by incubating at 35°C for a time period until the visual appearance of enough spores of *R. oligosporus* on PDA and was stored at 4°C until use. The optimum age of inoculum was determined by observing the appearance of *R. oligoropus* spores on inoculated PDA plates. Spores started appearing after 48 h, and enough spores could be seen after 72 h. Thus, a 72-hour-old inoculum of *R. oligosporus* was used for making the final concentration. For inoculation, the spore disruption method was used; a sporulated plate was flooded with 20 mL of sterile distilled water, and spores from the plate were brought into the water using a disposable spreader. The suspension was then set to reach a final concentration of 9.92×10^6 spores/mL by spore count using a hemocytometer.

Preparation of culture media

For optimization of process parameters for enhanced SCP production by fermentation of rice polishing, two comparative media, that is, rice polishing and control media, were prepared at pH 5.5. The control media was prepared with the composition 4% D-glucose, 2% peptone, 1% yeast extract, 0.05% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, and 0.05% NaCl in required volume of distilled water, and the rice polishing medium was prepared with the composition 10% rice polishing, 0.5% yeast extract, 0.05% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, 0.05% NaCl, and distilled water. Media were then sterilized by autoclaving for 20 min at 121°C and 15 psi.

Production of single-cell protein through liquid state fermentation

A 50 mL of autoclaved rice polishing and control media were transferred into sterile 250 mL Erlenmeyer flasks separately in triplicate under sterile conditions. The medium in each flask was inoculated with 2.5 mL (5% υ/υ) of *R. oligosporus* spore (9.92×10^6 spores/mL) suspension. The flasks were then incubated in a shaking incubator at 120 rpm for 96 h at 35°C. After fermentation, the fungal broth cultures were filtered through autoclaved filter paper (Whatman #41), and the fungal residue obtained was washed twice with distilled water to remove remaining broth medium that was first dried between sterile filter paper sheets and then in an oven at 60°C for 4 h to get constant weight. The dried biomass was then ground to pass through a 40-mesh sieve to be analyzed for CP content. The mean dry weight of biomass (g/L) was measured, and CP content (% of protein content in dry biomass) was quantified through the Kjeldahl method using factor 6.25 (Dharumadurai *et al*., 2011).

Optimization of process parameters in comparison with the control medium

For enhanced SCP production in rice polishing medium compared to that in the control medium, various fermentation conditions, such as substrate concentration (2, 4, 6, 8, 10, 12%), temperature (25, 30, 35, 40°C), initial medium pH (4.0, 4.5, 5.0, 5.5, and 6.0), inoculum size (5, 10, 15, 20%), fermentation period (24, 48, 72, 96, 120 h), and concentration of supplements (KH_2PO_4, $MgSO_4.7H_2O$, NaCl, and yeast extract) in medium, were optimized. Optimum conditions were acquired by the OFAT scheme, which is "one factor at a time" by keeping the other variables constant (Kheiralla *et al*., 2018). The constant values of the parameters while optimizing one factor at a time are substrate concentration 10%, temperature 35°C, pH 5.5, inoculum size 5% (υ/υ), fermentation period 96 h, and concentration of supplements in the substrate medium (0.5% yeast extract, 0.05% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, 0.05% NaCl). The mean dry biomass and the CP contents were estimated at every step of each optimization, and the results were compared with the control medium (glucose) to study the potential of rice polishing as a substrate for SCP. All optimization experiments were conducted in triplicate following the liquid-state fermentation method as described earlier. In the end, confirmatory experiments for SCP production were performed in triplicate with optimized process conditions in rice polishing and glucose (control) medium to validate the effect of optimization on dry biomass and protein content.

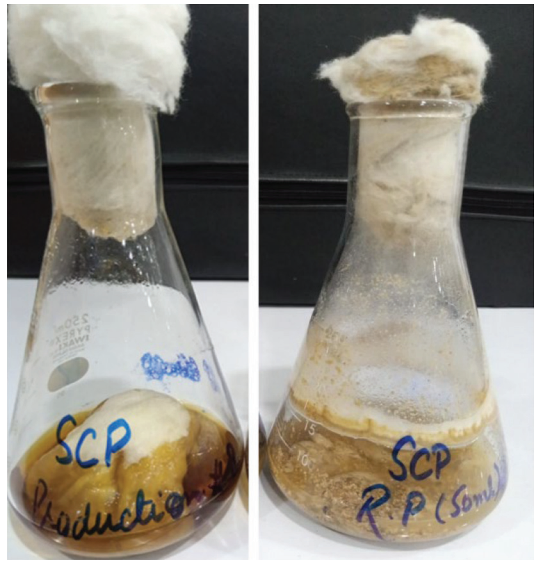

Figure 1. Single-cell protein production in control (glucose) medium and rice polishing (substrate) medium.

Analysis of cost-effective production of singe-cell protein in rice polishing medium

The economic potential of the SCP production in rice polishing medium was estimated by calculating prices for the ingredients used in the medium as compared to those of ingredients used in the control medium, with reference to the potential substrate medium for a higher amount of SCP production as compared to that of the control medium. The time utilized and the expenditure of energy during the processes were the same for both media; they were not included in the analysis because the process was optimized based on only the different growth conditions, including the different composition of the rice polishing medium as compared to that of the specific composition of the control medium.

Statistical analysis

The obtained data was statistically analyzed by taking rice polishing, control media, and fermentation parameters as fixed factors or independent variables and dry biomass and protein content as dependent variables. The effects of parameters on the yield of dry biomass and protein content within the two media were expressed as means of three replicates in each experiment, and the significance of the effects was estimated through one-way ANOVA using SPSS 15. The mean differences were categorized as statistically significant at P-value < 0.05. The least significant difference (LSD) between the effects of change in each variable was compared based on observed means through post hoc tests in SPSS.

Results and Discussion

Proximate composition of rice polishing

The biochemical composition of rice polishing, as shown in Table 1, was estimated to evaluate its nutritive value, which makes it a potential substrate to replace costly commercial culture media for SCP production. Several studies have been reported employing AOAC methods for the proximate analysis of different agricultural wastes that were used in the production of SCP through microbial fermentation of these wastes (Ibrahim Rajoka *et al*., 2004; Kumari *et al*., 2018; Maliki *et al*., 2023; Thiviya *et al*., 2022).

Optimization of fermentation parameters for enhanced single-cell protein production

The process parameters as mentioned above, were optimized for enhanced SCP production by liquid-state fermentation of rice polishing using *R. oligosporus*. For optimization, fermentation was done in rice polishing and glucose (control) medium, comparatively to study the potential of rice polishing as substrate. The effects of these parameters were studied through the estimation of dry biomass and CP content.

Effect of initial medium pH on single-cell protein production

The initial pH of the growth medium is a very important variable to be considered for the optimization of enhanced production of SCP by rice polishing, as compared to that of the control medium. A range of pH (4.0, 4.5, 5.0, 5.5, and 6.0) was checked to identify the optimum pH for the maximum yield of dry biomass and CP content. The results of this study showed that for rice polishing medium, maximum yields of dry biomass (21.65 ± 1.14) and protein content (48% ± 1.30) were recorded at pH 5.5, which are almost comparable to that of control medium that showed maximum yield of biomass (20.35 ± 1.21 g/L) at pH 5.0. A nonsignificant decrease of 0.06 g/L in yield at pH 5.5 was recorded, and the highest protein content (49.5% ± 1.07) was observed at pH 5.5. Beyond the optimum pH of 5.5, a clear and fast decrease in biomass and protein content was observed in both rice polishing and control medium because the metabolic activities of *R. oligosporus* might be affected at pH values other than the optimum pH (Figure 2).

Table 1. Chemical composition of substrate (rice polishing).

S. No.	Constituents	
1	Moisture content (%)	5.55 ± 0.99
2	Ash content (%)	8.33 ± 1.02
3	Crude fat (%)	9.69 ± 1.12
4	Crude protein (%)	13.94 ± 1.24
5	Carbohydrate (%)	63.49 ± 1.88
6	Fiber content (%)	0.20 ± 0.68
7	Metabolizable energy (kcal/100 g)	396.93 ± 6.5

Results shown in the third column of the table are the mean ± standard deviation of three replicates of % values for each constituent of substrate (rice polishing).

The cultivation conditions of *R. oligosporus* have been reported in several studies for SCP production, and different optimum pH values of the medium have been reported depending on the composition of the culture medium and the type of substrate used for SCP production. For example, in a study of utilization of carbon and nitrogen by *R. oligosporus*, the optimum pH was found to be 5.0 (Sorenson and Hesseltine, 1966). In another report of SCP production by natural rubber wastes using *R. oligosporus*, the optimum pH of the medium was reported as 4.0 (Mahat and MacRae, 1992). Similarly, there are different reports about the optimum pH for SCP production by rice polishing depending upon the organisms used (Ahmed *et al*., 2017; Ibrahim Rajoka *et al*., 2004). The optimum pH of 5.5, similar to our study, has also been reported in literature, including a report where *Neurospora intermedia, Rhizopus sp.*, *Aspergillus oryzae, Fusarium venenatum, and Monascus purpureu*s were used to produce biomass protein at this pH (Ferreira *et al*., 2014).

Effect of incubation temperature (°C)

Incubation temperature is another important process variable that affects the yield of dry biomass and CP content in rice polishing and control medium. Different incubation temperatures (25, 30, 35, 40°C) were checked for estimating the highest yield of SCP in rice polishing medium at pH 5.5 (optimized in the first step). Figure 3 shows the effect of incubation temperature on SCP production. The maximum values of dry biomass (19.57 ± 0.86 g/L) and CP content (48.5 ± 1.15%) were observed at 30°C in rice polishing medium, and in case of control medium, the highest yield of dry biomass (21.2 ± 0.76 g/L) and highest protein content (50 ± 1.15) were recorded at 35 and 30°C, respectively. It was observed that dry mass values at 30 and 35°C showed a nonsignificant decrease of 0.37 and an increase of 0.2 g/L for rice polishing and control medium, respectively. Thus, in the current study, 30°C was found to be the optimum incubation temperature for rice polishing, as well as the control medium, for enhanced production of dry biomass and protein content.

A significant decrease in the yield of biomass and protein content was observed when the temperature increased above 35°C (Figure 2), which may be caused by the inactivation of metabolic enzymes and the disruption of cellular processes at high temperatures. At lower temperatures, lower biomass and protein contents were observed, perhaps as a result of impaired membrane function that prevented the uptake of important substrates. The optimum incubation temperature for the growth of *R. oligosporus* has been reported as 30, 35, and 37°C (Abdullahi *et al.*, 2021; Ibrahim Rajoka *et al.*, 2004; Mahat and MacRae, 1992; Mahnaaz *et al.*, 2009; Reihani and Khosravi-Darani, 2019), while the optimum temperature for SCP production by *R. oligosporus* in most of the studies was found to be ~28°C. The increase in incubation temperature from 35°C resulted in a significant decrease in the *R. oligosporus* biomass and protein content (Mahat and MacRae, 1992). In the present study, the same trend was observed with optimum temperature 30°C for CP content, but in the case of biomass, a nonsignificant decrease was observed from 30 to 35°C, and a significant decrease was recorded after 35°C. In another study of SCP production from rice polishing using *Candida utilis,* the optimum temperature for maximum biomass and protein content was found to be 35°C (Rajoka *et al.*, 2006).

Effect of substrate concentration (% w/v)

Substrate concentration used in the culture medium can significantly affect the yield of biomass and protein content. Thus, different percentages (w/v) of rice polishing were used in the medium to check the optimum concentration for maximum SCP production at 30°C incubation temperature and 5.5 pH of the medium (optimized in the first two steps). According to the study results (Figure 4),

(A) (B)

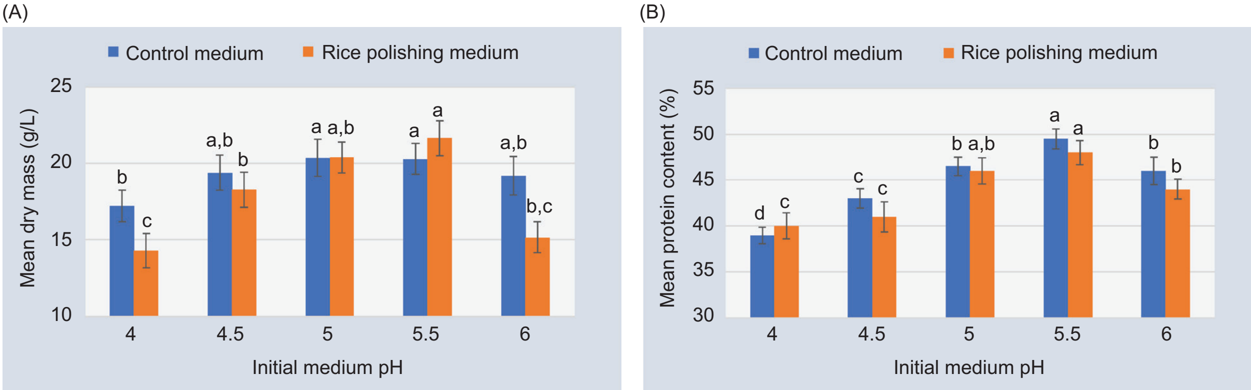

Figure 2. Effect of pH on single-cell protein production by rice polishing as compared to the control medium. (A) Effect of pH on dry biomass; (B) Effect of pH on the protein content. Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences in dry biomass and protein content in rice polishing and the control medium.

(A) (B)

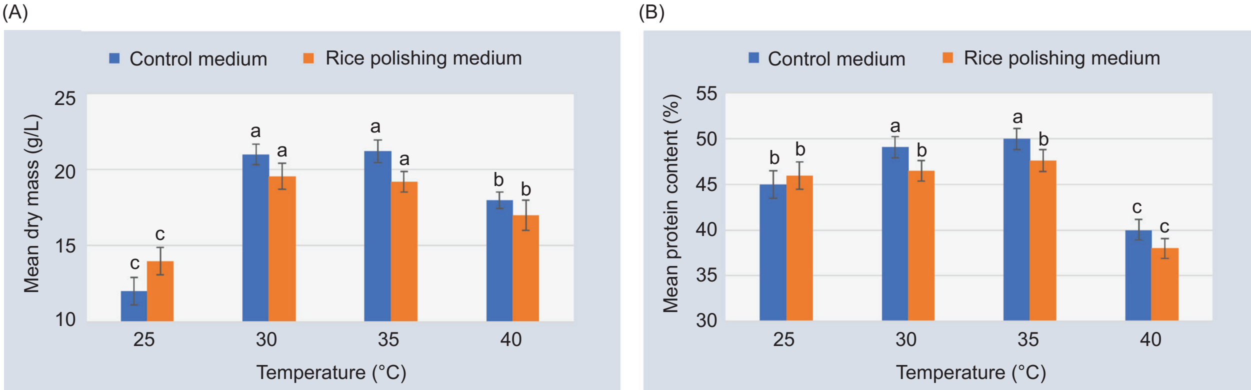

Figure 3. Effect of temperature on single-cell protein production in rice polishing medium as compared to the control medium. (A) Effect of temperature on dry biomass; (B) Effect of temperature on the protein content. Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences in dry biomass and protein content in rice polishing and the control medium.

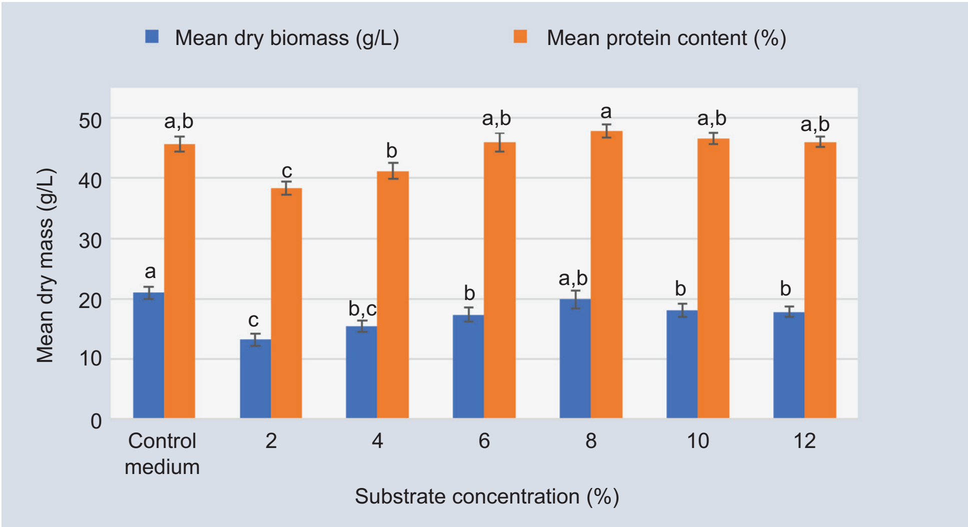

Figure 4. Effect of substrate concentration (rice polishing) on single-cell protein production compared to the control medium: Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences of dry biomass and protein content at different substrate concentrations in rice polishing medium as compared to that of the control medium with fixed composition.

the highest dry biomass (19.89 ± 1.52 g/L) and protein content (47.87 ± 1.09%) were recorded for 8% (w/v) of rice polishing in comparison with biomass (21± 1.02 g/L) and protein content (45.67± 1.19%) in control medium with fixed concentration of glucose (4%) and peptone (2%). Fungal biomass and protein content did not increase with further increases in substrate concentration, and a slight decrease in values was observed. Thus, in this study, 8% (w/v) rice polishing has been proved to be a potential carbon source for fungal biomass production. Rice polishing is a very abundant, cheap, and easily available agricultural waste that can easily replace a costly carbon source (glucose) in the medium for economic production of microbial biomass protein. In the literature, different concentrations of rice polishing, such as 5, 9, and 10% (w/v), have been reported for the highest yield of SCP depending upon the different organisms used. For example, using 10% rice polishing for SCP production by *R. oligosporus* and *C. utilis* as coculture was reported in the study by Nadeem *et al.* (2016). Similarly, in the kinetic study of SCP production from rice polishing using *C. utilis*, 9% substrate was used in minimal medium (Rajoka *et al.*, 2006); the same concentration of rice polishing was used in a similar study using *C. utilis* (Ibrahim Rajoka *et al.*, 2004). Maximum fungal biomass protein yield from *Trichoderma harzianum* was obtained using 5% (w/v) rice polishing (Ahmed *et al.*, 2017).

Effect of inoculum size (% v/v)

To find the optimum inoculum size for enhanced production of SCP, 9.92 × 106 spores/mL and different inoculum percentages (5, 10, 15, 20% v/v) were used. Figure 5 shows that the maximum yields of dry biomass (21.3 ± 10.89 g/L) and protein content (50 ± 1.25%) were observed for an inoculum size of 5%, which is comparable with the maximum values of biomass (22.5 ± 1.12 g/L) and protein content (51.5 ± 1.22%) in control medium. A gradual decrease in biomass and protein contents was observed as the inoculum size increased. The addition of inoculum to liquid medium higher than 5% might result in an increased demand for nutrients and available oxygen in controlled culture conditions, making it difficult for the fungus to grow fully in a scarce environment and to function properly for normal production of protein content with limited required metabolites and enzymes. Several studies have reported using different optimum percentages or ratios of inoculum size (Yalemtesfa *et al.*, 2010). For example, the maximum production of protein content from *C. utilis* was obtained at an inoculum size of 7.5% (v/v) (Li *et al.*, 2009). In another study on the biosynthesis of single-cell biomass of *C. utilis* using fruit waste extract, maximum yield of biomass was attained with 4% inoculum size (Munawar, 2010). In the optimization process for SCP production from *C. utilis* in solid-state fermentation, 10% (v/v) inoculum size was found to show the maximum biomass (Irfan *et al.*, 2011). In a similar study estimating the kinetics of batch SCP production from rice polishing with *C. utilis*, the highest biomass protein was obtained with 10% (v/v) inoculum size (Rajoka *et al.*, 2006). 3% (v/v) inoculum size was used to obtain maximum biomass of *A. oryzae* using deoiled rice bran (Ravinder *et al.*, 2003). Maximum biomass of *A. niger* from rice bran was obtained using 2% (v/v) inoculum size (Oshoma and Ikenebomeh, 2005).

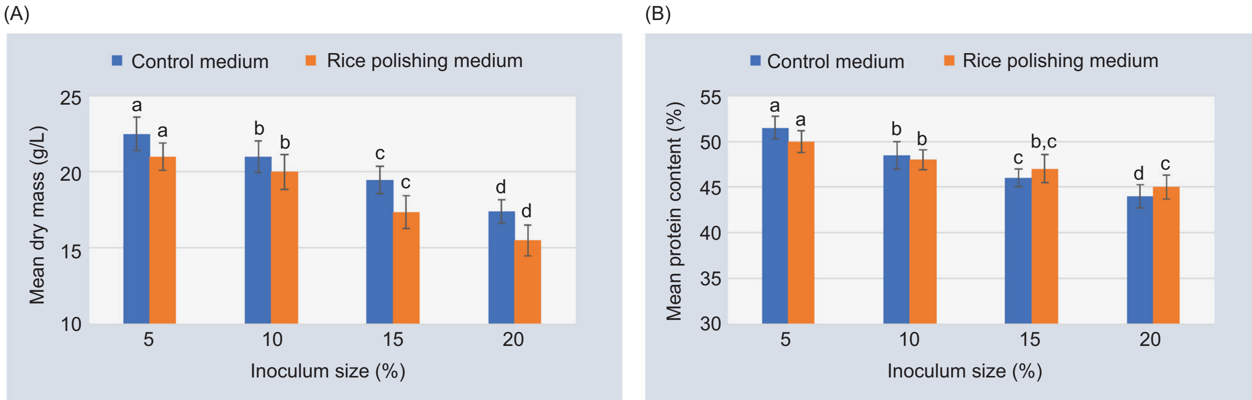

Figure 5. Effect of inoculum size (*Rhizopus oligosporus*) on single-cell protein production by rice polishing as compared to the control medium. (A) Effect of inoculum size on dry biomass; (B) Effect of inoculum size on the protein content. Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences in dry biomass and protein content in rice polishing and the control medium.

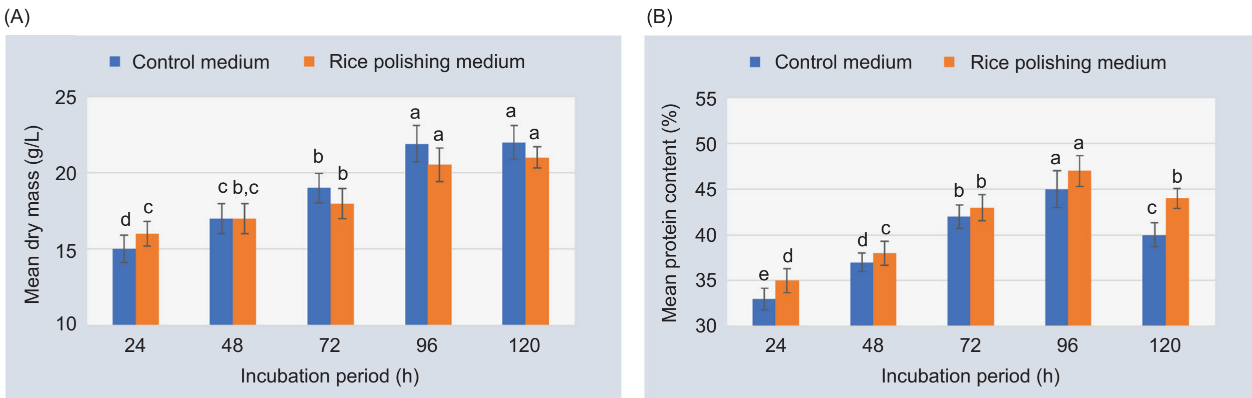

Figure 6. Effect of incubation period on single-cell protein production by rice polishing as compared to the control medium. (A) Effect of incubation period on dry biomass; (B) Effect of incubation period on the protein content. Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences in dry biomass and protein content in rice polishing and the control medium.

Effect of incubation time (h)

Different incubation periods (24, 48, 72, 96, and 120 h) were studied to identify the optimum time of incubation for maximum SCP production. Figure 6 shows that the highest yields of dry biomass, 21.05 ± 1.1 g/L and 22.14 ± 1.7g/L, were observed after 120 h of incubation in rice polishing and control medium, respectively, with very little increase from 96 to 120 h (almost constant). But, the maximum protein content, 47 ± 1.3% in rice polishing medium and 45 ± 2.0% in control medium, was obtained after 96 h. In that way, biomass was found to be almost constant after 96 h, and protein content decreased significantly because with the passage of time, protein at a certain temperature might start to degrade by losing its structural integrity and potential to function properly. Thus, 96 h of incubation was decided to be the optimum incubation period for enhanced SCP production in the present study. Similar studies in the literature have been reported with different incubation periods for maximum production of SCP, depending on the type of organisms and substrates used. For example, the highest single-cell biomass of *C. utilis* using fruit waste extract was recorded after 96 h (Munawar, 2010). Similarly, in the optimization of the production of single-cell biomass of *C. utilis* from wheat bran, the maximum biomass was recorded after 96 h (Irfan *et al.*, 2011). In another study, the maximum yield of fungal biomass protein from rice polishing using *T. harzianum* was obtained after 72 h of incubation (Ahmed *et al.*, 2017). The highest SCP yield was obtained after 72 h while studying *A. oryzae* for the production of SCPs from deoiled rice bran (Ravinder *et al.*, 2003). Similarly, in the production of SCP-enriched feed supplement through waste conversion by *Candida sp.*, maximum SCP was obtained after 72 h (Adoki, 2002).

Effect of the concentration of supplements in the medium

In this study, different concentrations of inorganic salts $MgSO_4.7H_2O$, KH_2PO_4, and NaCl, as well as different concentrations of yeast extracts, in rice polishing medium were tested to get optimal fungal biomass protein production by fermentation of *R. oligosporus*, by changing the concentration of one supplement at a time and keeping other supplements constant with values 0.5% yeast extract, 0.05% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, and 0.05% NaCl. Figure 7 shows that the highest yields of biomass and protein content were obtained at optimum salt concentrations of 0.05% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, and 0.075% NaCl in rice polishing medium, which is comparable to biomass (20.2 ± 1.02 g/L) and protein content (46 ± 1.19%) in the control medium containing fixed concentration of salts and yeast extract (Figure 6b).

In the literature, several similar studies have been conducted for the optimization of enhanced SCP production through salt concentration in the culture medium. For example, the study by Thiviya *et al.* (2022) on the production of SCP from fruit peel wastes using palmyrah toddy yeast, where the optimum concentrations of 0.1% KH_2PO_4, 0.05 % $MgSO_4.7H_2O$, 0.01% NaCl, and 0.1% $CaCl_2$ were used in fruit peel medium for enhanced production of biomass and protein contents, showed results similar to ours. In another study, salt concentrations of 0.5% $(NH_2)2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% NaCl, and 0.01% $CaCl_2$ were used in pineapple waste medium for enhanced production of SCP using yeast (Dharumadurai *et al.*, 2011). The maximum production of biomass protein by fermentation of a mixed culture of *Arachniotus sp*., and *C. utilis* using corn stover was observed at a much lower optimum concentration of 0.0075% $CaCl_2.2H_2O$, 0.005 % $MgSO_4.7H_2O$, and 0.01% KH_2PO_4 in the medium because of the different medium conditions due to mixed microbial culture (Ahmed *et al.*, 2010). In most of the studies, the concentrations of KH_2PO_4, $MgSO_4.7H_2O$, and $CaCl_2$ are optimized in the medium for enhanced SCP production; in some studies, both $CaCl_2$ and NaCl are used in addition to the other salts. However, in the present study, only NaCl was used in addition to KH_2PO_4 and $MgSO_4.7H_2O$ to minimize the cost of the medium, because NaCl is cheaper and is reported to be more effective in increasing the mycelial growth than $CaCl_2$. $CaCl_2$ shows an average effect on fungal biomass production. However, after a certain concentration, NaCl starts inhibiting the growth of fungal biomass (Boumaaza *et al.*, 2015), due to which its concentration in the medium needs to be optimized.

Figure 8 shows the results of optimization of the supplemented amount of yeast extract in rice polishing medium compared to the control medium. As shown in the figure, an instant increase in biomass and protein content was noticed when rice polishing medium was initially supplemented with 0.02% yeast extract. While increasing the supplemented amount of yeast extract from 0.02 to 0.1%, no notable increase in dry biomass and protein content was observed. The maximum biomass (19.0 ± 1.09 g/L) was obtained with 0.1% yeast extract and highest protein content (47.21 ± 0.96%) with 0.08% yeast extract in the rice polishing medium compared to the biomass (20.2 ± 1.02 g/L) and protein content (46 ± 1.19%) in control medium with fixed amount of supplemented yeast extract (1%). As commercial yeast extract is costly, the optimum amount of yeast extract in rice polishing medium for enhanced SCP production was decided to be 0.08%. There are similar reports on the optimized supplementation of yeast extract in the medium for enhanced SCP production, compared to the control medium. For example, in the production of SCP from soy molasses using *Candida tropicalis*, a trend similar to the present study was observed with a prominent increase in biomass

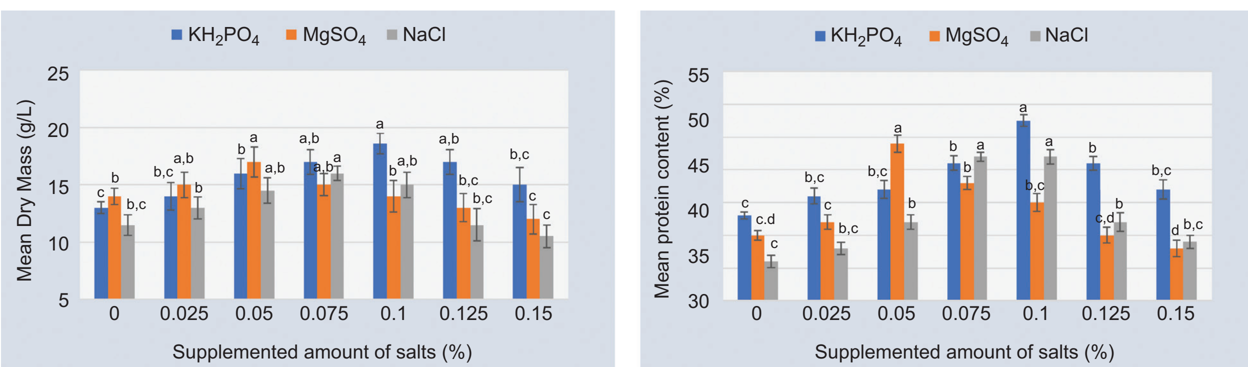

Figure 7. Effect of concentration of salts in rice polishing medium on single-cell protein production: Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences in dry biomass and protein content as a result of different concentrations of KH_2PO_4, $MgSO_4.7H_2O$, and NaCl in rice polishing medium.

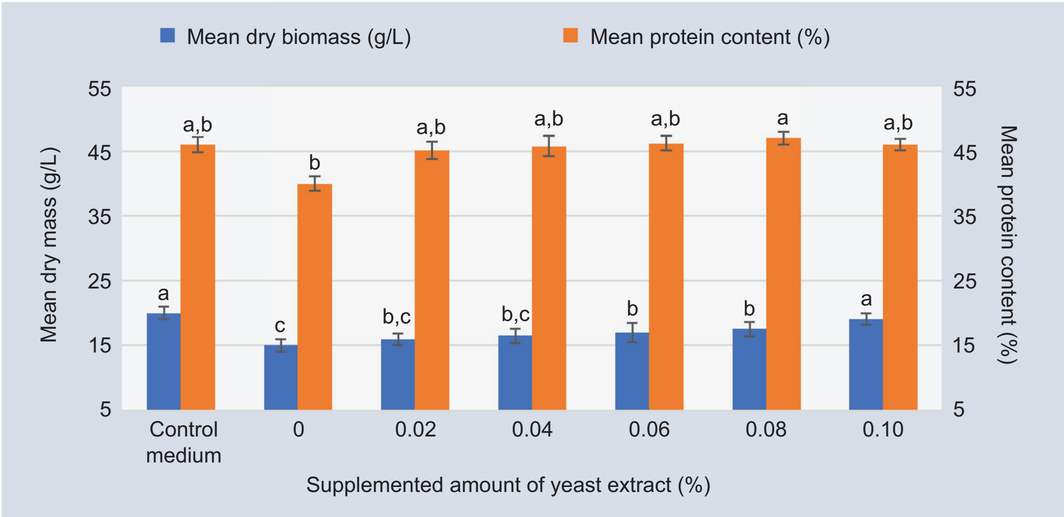

Figure 8. Effect of concentration of yeast extract in rice polishing medium on single-cell protein production as compared to the control medium. Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. Lowercase letters indicate the significant differences in dry biomass and protein content as a result of different concentrations of yeast extract in rice polishing medium, as compared to that of the control medium with a fixed concentration of yeast extract in the media composition.

and crude protein with initial supplementation of 0.02% yeast extract, and further addition of yeast extract did not show any significant increase. Thus, 0.02% yeast extract was decided to be the optimum amount of yeast extract in the medium (Gao *et al*., 2012). But in the present study, although an increase in protein biomass was not significant from 0.02–0.1% yeast extract, 0.08% was decided to be the optimum amount with maximum protein content.

Effect of optimized process parameters on dry biomass and protein content

Final confirmatory experiments were done in triplicate to evaluate the effect of optimized fermentation variables on the yield of dry biomass and protein content produced by rice polishing using *R. oligosporus* as compared to that of the control medium. The optimum conditions including substrate concentration 8%, temperature 30°C, pH 5.5, inoculum size 5%, fermentation period 96 h, and optimum concentration of supplements (0.05% $MgSO_4.7H_2O$, 0.1% KH_2PO_4, 0.075% NaCl, and 0.08% yeast extract) in the rice polishing medium were used in fermentation process on this step for the production of SCP. Figure 9 shows that the optimization of process parameters of rice polishing medium resulted in biomass production (19.5 ± 1.93 g/L) significantly lower ($P < 0.05$) than the control medium (22.36 ± 2.13 g/L), but a significantly higher protein content (49.5 ± 0.9%) was recorded in rice polishing medium than that of the control medium (46.12 ± 0.6%). These results suggested that rice polishing and *R. oligosporus* could be used to produce biomass and protein content that is comparable to the control medium (glucose).

Estimation of cost-effective impact of rice polishing for single-cell protein production

Table 2 shows a huge cost-effective impact of rice polishing medium optimized through the present study as compared to the control (glucose) medium. In substrate medium, the cost was mainly managed by replacing glucose and peptone as carbon and nitrogen sources, respectively, by a much cheaper source rice polishing in the substrate medium because proximate analysis of rice polishing shows a crude protein content of 13.94% ± 1.24 and carbohydrate content of 63.49% ± 1.88 as nitrogen and carbon sources, respectively, for SCP production (Table 1). Similarly, the optimized quantity of supplements in the medium contributed to the low-cost production of SCP using rice polishing medium, as shown in the table.

Conclusions

The agricultural waste, rice polishing, is a highly economical and easily available source of energy with high nutritive value of CP, CF, FC, and carbohydrate contents. Its potential to be used as a substrate for the production of SCP was evaluated using the fungus *R. oligosopurus*

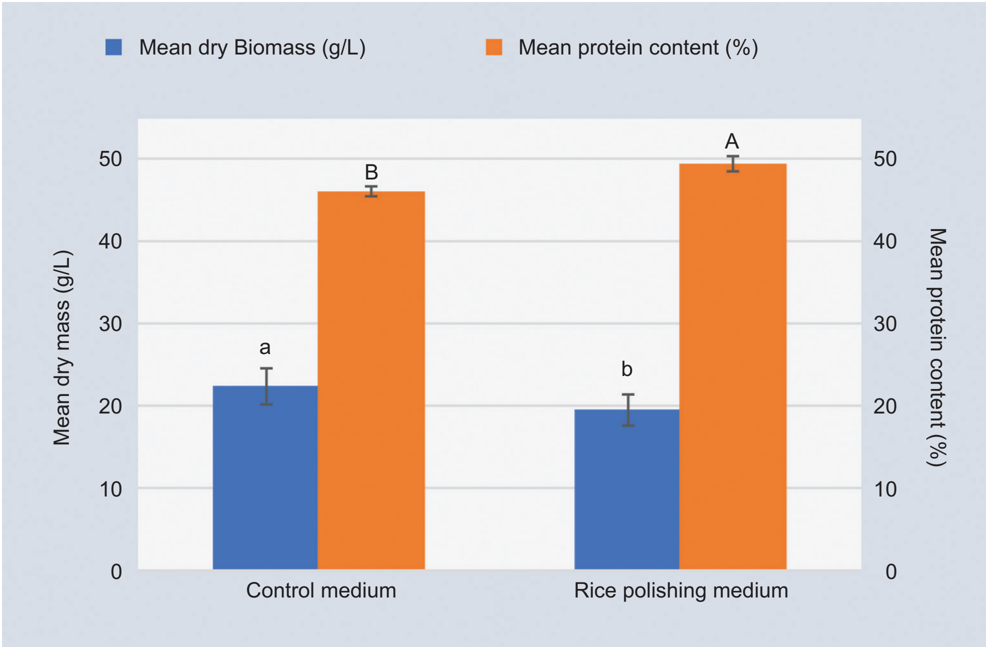

Figure 9. Effect of optimization on dry biomass and protein content in rice polishing medium in comparison with control medium: Results in the graph are the mean of experiments in triplicate (n = 3) ± standard deviation, which differ significantly at P < 0.05. The lowercase letters indicate the significant differences in dry biomass and protein content as a result of optimized conditions of rice polishing medium as compared to those of the control medium.

Table 2. Analysis of cost-effective single-cell protein production in rice polishing medium compared to the control medium.

		Glucose	Rice polishing	Peptone	Yeast extract	$MgSO_4$	KH_2PO_4	NaCl	Total price
Prices ($)/g/ 100 mL	Control medium	0.059472	0	0.1368	0.054	0.00054	0.001198	0.0000245	0.252034
	Rice polishing medium	0	0.01152	0	0.00432	0.00054	0.001198	0.0000367	0.017614

Results in the table show a comparison of prices (million) of different ingredients used in rice polishing as well as in the control (glucose) medium in grams/100 mL of each medium. The total price for each medium is also compared in the table.

in the present study. *R. oligosporus*, being a domesticated strain with no production of mycotoxin metabolites, unlike other fungi, produces SCP that can be highly nutritious and safe for health. For enhanced and low-cost production of SCP in rice polishing medium using fungus in comparison with control (glucose) medium, fermentation parameters can be optimized. The maximum final yield of dry biomass and protein content was obtained in rice polishing medium with optimum substrate concentration of 8%, incubation temperature 30°C, initial medium pH 5.5, inoculum size 5%, incubation period, and supplemented amount of 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.075% NaCl, and 0.08% yeast extract in the medium. The yield of this dry biomass and protein content with significantly low cost and minimum possible ingredients in rice polishing medium (as shown in Figure 9) was comparable to the biomass and higher than the protein content in costly commercial glucose medium. This optimized process of SCP production from rice polishing using *R. oligosporus* can further be used for large-scale protein production in bioreactors and to evaluate SCP for its utilization as a nutritive supplement in feed for livestock, poultry, and even for humans and in other industrial and commercial processes.

Data Availability Statement

All data produced or generated during the study have been given in the manuscript.

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Conflicts of Interest

The authors do not have any conflicts of interest to disclose.

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