AJLS

Asean Journal of Life Sciences, Vol 4(1), 2026, pp 31-42

Accepted date: 23 March 2026
Published date: 30 June 2026

Improved Protein Recovery from *Candida utilis* through Combined Enzymatic and Mechanical Disruption Using Pineapple Waste

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Abstract

This study investigated a sustainable strategy to improve intracellular protein recovery from *Candida utilis* cultivated on pineapple waste by integrating enzymatic pre-treatment with mechanical cell disruption. The rigid yeast cell wall, composed mainly of β-glucans, mannoproteins, and chitin, limits efficient protein extraction. Mechanical disruption using glass beads and enzymatic pre-treatment with cellulase and protease were evaluated individually and in combination. Sequential enzyme treatments combined with glass bead disruption achieved the highest protein recovery (12.2%), representing approximately 19.6% and 43.5% increases over standalone mechanical and individual enzymatic treatments, respectively. However, this improvement was not significantly different ($p > 0.05$) from the simpler cellulase–glass bead treatment, which achieved comparable protein recovery within a substantially shorter processing time (18 hours versus 36 hours), highlighting its greater operational efficiency and industrial potential. In addition, pineapple waste was successfully utilized as a low-cost fermentation substrate, supporting agro-industrial waste valorization and improving process sustainability. Overall, the integration of enzymatic pre-treatment with mechanical disruption provides an effective and scalable approach for sustainable yeast protein extraction with potential applications in the food, pharmaceutical, and biotechnology industries.

Keywords: *Candida utilis*; Cell disruption; Enzymatic hydrolysis; Pineapple waste; Protein extraction

INTRODUCTION

Candida utilis (*C. utilis*) is a valuable source of single-cell protein (SCP) which can produce from fruit waste extract, with potential for use in feed supplements to improve protein deficiency and malnutrition (Munawar et al., 2010). *C. utilis* can also produce single cell protein from wheat bran using solid state fermentation, potentially improving nutritional problems associated with protein deficiency (Irfan et al., 2011). Yeast extracts, derived from *C. utilis* and other yeasts, primarily derived from spent brewer's yeast, is a nutrient-rich substance containing nucleotides, proteins, amino acids, carbohydrates, and essential trace elements. Its production is cost-effective due to the readily available raw material. Owing to its versatile biochemical composition, yeast extract is extensively applied in animal nutrition, food additives and flavor enhancers, cosmetic formulations, and as a nutrient source in microbial fermentation. Nevertheless, its full biotechnological and industrial potential remains underexploited (Tao et al., 2022).

Despite advancements in extraction techniques, the robust yeast cell wall, primarily composed of β-glucans, mannoproteins, and chitin, continues to present a significant challenge to the efficient extraction of intracellular proteins (Liu et al., 2016) The intricate and highly cross-linked architecture of these components contributes to its mechanical strength and impermeability, necessitating disruptive methods for effective protein release. Consequently, effective yeast cell wall disruption is paramount for maximizing protein recovery and achieving optimal yields of intracellular components, including yeast extract (Tao et al., 2022; Zainuddin et al., 2021).

A diverse array of methods has been employed for yeast cell wall disruption, encompassing mechanical methods such as bead milling, high-pressure homogenization, and ultrasonication; enzymatic approaches utilizing enzymes like glucanases, proteases, and lytic enzymes; and chemical treatments including alkali, acid, or detergent-based lysis (Zainuddin et al., 2021). While mechanical methods are generally effective, they often involve high energy consumption, can generate significant heat potentially leading to protein denaturation, and may not be universally applicable across all yeast strains. Enzymatic methods provide a gentler approach for cell disruption, which is crucial for minimizing protein denaturation and preserving bioactivity (Jacob et al., 2019; Tang et al., 2024). Previous studies demonstrated that disruption efficiency is highly dependent on yeast wall composition and the disruption technique employed. Bzducha-Wróbel et al., (2014) reported significant variations in β-glucan recovery among bead milling, autolysis and chemical extraction methods. More recently, Mantri et al. (2025) emphasized the importance of integrating enzymatic and mechanical strategies to overcome the recalcitrant fungal cell wall structure and enhance intracellular product recovery.

However, these methods are often characterized by slower reaction rates, higher operational costs due to enzyme expenses, and potential inconsistencies depending on the cell type and precise control required over reaction conditions (Mantri et al., 2025; Tao et al., 2022). To overcome the limitations of individual methods, combining enzymatic and mechanical approaches has shown significant promise. This synergistic strategy typically involves an enzymatic pre-treatment to weaken the robust yeast cell wall, followed by a less intensive mechanical disruption to effectively release intracellular contents (Mantri et al., 2025). This

combined approach not only reduces the overall energy input required for mechanical disruption but also leads to significantly improved protein yield and purity (Mantri, et al., 2025; Tao et al., 2022).

Despite numerous studies investigating mechanical, enzymatic and autolytic disruption techniques for yeast biomass processing, limited information is available regarding the sequential application of protease and cellulase in combination with glass bead disruption for intracellular protein recovery from *C. utilis* cultivated on pineapple waste substrate. Furthermore, studies integrating agro-industrial waste valorisation with optimized yeast cell disruption strategies remain scarce. Therefore, this study aimed to optimize enzymatic, mechanical and combined enzymatic–mechanical disruption approaches to improve protein extraction efficiency and develop a sustainable process for yeast extract production. Furthermore, this research explores the use of pineapple waste as a cost-effective growth medium for *C. utilis*. Pineapple waste, which is abundantly generated in Malaysia from processing and harvesting activities (Azman & Romli, 2024; Gopinathan et al., 2024) represents a highly promising and low-cost substrate for yeast cultivation. Its utilization aligns strongly with the principles of sustainable bioprocessing and the valorization of agro-industrial by-products, contributing to a circular bioeconomy (Gopinathan et al., 2024; Puspitasari et al., 2024). The utilization of such agro-industrial waste not only reduces production costs but also contributes to environmental sustainability by minimizing waste disposal issues.

METHODOLOGY

Yeast Cultivation

C. utilis was obtained from the Culture Collection Centre of Universiti Teknologi MARA (Shah Alam, Malaysia). A preculture was prepared by inoculating *C. utilis* into 50 mL of pineapple waste broth (prepared according to Liong et al., 2002) and incubating at 30°C for 24 hours at 100 rpm. This preculture was then inoculated (7.8% v/v) into a 1.5 L fermenter containing 500 mL of pineapple waste medium and cultivated at 30°C for 30 hours at 100 rpm. The composition of the pineapple waste medium was analyzed to determine its sugar content and other nutritional components relevant for yeast growth.

Cell Disruption Methods

Mechanical Degradation Using Glass Beads

Approximately 5 g of glass beads (0.25–0.65 mm, Sigma -9268) were added to a 6 mL yeast suspension (40% w/v) in a 50 mL conical flask (prepared in duplicate). The mixture was incubated at 50°C with agitation at 250 rpm for 24 hours using an incubator shaker (Hotech, Japan). Samples were collected at specific time intervals (0, 2, 4, 6, 8, 12, 18, and 24 hours). From each flask, 2 mL was taken for cell disruption analysis, while the remaining 4 mL was centrifuged (9000 rpm, 10 minutes) to obtain a supernatant for soluble protein analysis.

Single Enzymatic Degradation With or Without Glass Beads

For enzymatic degradation without glass beads, protease (Neutrase 1.5MG) and cellulase (Celluclast 1.5 L FG) were added at concentrations of 0.5% and 1.0% to a 40% (w/v) yeast suspension, prepared in a buffer at the optimum pH of each enzyme (pH 6.5 for protease and pH 5.0 for cellulase). The reaction was carried out in a 50 mL conical flask (in duplicate) at

50°C with agitation at 250 rpm for 24 hours. Samples were collected and analyzed similarly to the mechanical degradation process. For enzymatic treatment with glass beads, the same procedure was followed, except 5 g of glass beads were added to enhance cell disruption.

Combination of Enzymatic Treatment with Glass Beads

To evaluate enzyme combinations for cell degradation, experiments were conducted with a fixed enzyme concentration of 0.5%, based on the previous optimum results, which showed no significant difference ($p>0.05$) between 0.5% and 1.0%. Two treatment approaches were tested:

a) Simultaneous Enzyme Addition

A 40% (w/v) yeast suspension was mixed with 5 g of glass beads in a 50 mL conical flask (in duplicate), and both enzymes (0.5% protease and 0.5% cellulase) were added at their respective optimum pH levels. The mixture was incubated at 50°C, 250 rpm for 24 hours. Samples were taken at 2 to 6-hour intervals and analyzed as described earlier.

b) Sequential Enzymatic Treatment

Two sequential treatments were tested:

Protease followed by cellulase (PC): 0.5% protease (pH 6.5) was added first, and the mixture was incubated for 24 hours. Then, the pH was adjusted to 5.7 before adding 0.5% cellulase.

Cellulase followed by protease (CP): 0.5% cellulase (pH 5.7) was added first and incubated for 18 hours. The pH was then adjusted to 6.5 before adding 0.5% protease.

All treatments were carried out in 50 mL conical flasks containing a 40% (w/v) yeast suspension in either phosphate buffer (pH 6.5 for protease or pH 5.7 for cellulase). Each flask contained 6 mL of yeast suspension and 5 g of glass beads. The incubation was conducted at 50°C with agitation at 250 rpm for a total duration of 42 hours. The 42-hour incubation period was selected to ensure completion of sequential enzymatic hydrolysis, allowing sufficient time for the first enzyme to weaken the yeast cell wall structure prior to the addition of the second enzyme. Prolonged incubation periods have been reported to enhance yeast cell wall degradation and facilitate intracellular component release during enzymatic and autolytic treatments (Middelberg, 1995; Reed & Nagodawithana, 1991). Samples were collected every 6 hours, centrifuged, and analyzed as described previously.

Analytical Methods

Intracellular Protein Content

Protein concentration was determined according to the method of Lowry et al. (1951). Protein content was expressed as mg protein/g dry cell weight.

Cell Disruption Percentage

The percentage of cell disruption was quantified using a hemocytometer and light microscopy. The number of intact cells was counted under a compound microscope at 400× magnification across at least five large squares, with three replicates performed per sample to ensure counting precision. The percentage of disrupted cells was calculated using the following formula:

$$\text{Percentage Disrupted Cells} = \frac{\text{Number of Disrupted Cells}}{\text{Number of Intact Cells+Number of Disrupted Cells}} X100\%$$

To maintain complete consistency with the data presented in the text, the percentage of remaining intact cells reported in Table 1 is expressed as:

$$\text{Intact Cells } (\%) = 100\% - \text{Cell Disruption } (\%)$$

This approach provides a clear, quantitative conversion between the direct microscopic counts and the cell disruption efficiency achieved across the different treatment strategies.

Total Soluble Solids (TSS)

Total Soluble Solids (TSS) was measured using a refractometer according to AOAC Official Methods of Analysis (AOAC International, 2023).

Microscopic Analysis

Cell morphology and integrity were assessed using a compound microscope. Samples were prepared by mixing cells with distilled water and observed at 400x to identify changes in cell shape, aggregation, or lysis before and after the degradation method. Meanwhile, for detailed visualization of cell wall structure, transmission electron microscopy (TEM) was used to visualize cell wall structure before and after treatment. Samples underwent standard preparation, including fixation, dehydration, resin embedding, and ultra-thin sectioning. Stained sections were then examined using TEM at appropriate magnifications (20,000x–100,000x) to observe changes in cell wall thickness, density, and ultrastructural damage after treatment.

Statistical analysis

Microscopic cell counts were performed in triplicate for each sample to improve counting precision. However, due to substrate constraints, the downstream protein extraction experiments were conducted in duplicate (n = 2). While duplicates provide an initial comparative baseline, this smaller sample size lowers the statistical power of the analysis; thus, the resulting statistical groupings should be interpreted with appropriate caution. All data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS version 27.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was employed to determine significant differences among treatments at $p < 0.05$.

RESULTS AND DISCUSSION

Mechanical disruption using glass beads resulted in a gradual increase in intracellular protein concentration over time, reaching a maximum at a specific time point (Table 1). At pH 6.5, mechanical treatment left only 11.8% of cells intact, corresponding to approximately 88.2% cell disruption. Protein extraction efficiency was also higher at pH 6.5 (10.2%) than at pH 5.0 (8.6%), indicating that near-neutral pH conditions enhanced protein recovery during mechanical disruption. This environmental dependency aligns with observations that yeast intracellular protein solubility and release dynamics fluctuate significantly across varying pH ranges, with neutral environments generally facilitating enhanced mass transfer and protein extractability over acidic settings (Ganeva et al., 2020; Mantri, 2025). Furthermore, these findings align with previous studies where mechanical disruption achieved significant protein

extraction but required high energy input and prolonged processing times (Ekpeni et al., 2016). Higher temperatures (e.g., 50°C and 60°C) appeared to enhance protein release compared to ambient temperature possibly due to increased cell wall weakening and protein solubility. However, excessively high temperatures could lead to protein denaturation, which needs to be considered.

Table 1 Summary of analysis results for each cell degradation treatment method under optimum conditions (mean ± SD, n = 2)

Treatment Method	*Time (hour)	Protein content (%)	TTS (%)	***% of intact cells (%)
1) Mechanical Cell Degradation				
• pH 5.0 (with glass beads)	24	8.6 ± 0.19^{d}	4.3 ± 0.07^{d}	22.4 ± 3.80^{c}
• pH 6.5 (with glass beads)	24	10.2 ± 0.04^{e}	4.1 ± 0.07^{d}	11.8 ± 0.65^{b}
• pH 5.0 (without glass beads)	24	2.2 ± 0.09^{a}	2.6 ± 0.00^{b}	82.4 ± 1.12^{g}
• pH6.5 (without glass beads)	24	2.9 ± 0.04^{ab}	1.4 ± 0.00^{a}	69.4 ± 6.58^{ef}
2) Individual Enzymatic Cell Degradation Without Glass Beads				
• Protease (0.5 %)	24	8.5 ± 0.03^{d}	2.5 ± 0.14^{b}	61.2 ± 3.31^{de}
• Protease (1.0 %)	24	3.9 ± 0.07^{bc}	3.1 ± 0.00^{c}	69.4 ± 6.52^{e}
• Cellulase (0.5 %)	24	8.5 ± 0.28^{d}	3.1 ± 0.00^{c}	54.9 ± 2.22^{d}
• Cellulase (1.0 %)	24	4.2 ± 0.34^{bc}	3.6 ± 0.14^{cd}	$77.4 \pm 6.51^{f g}$
3) Individual Enzymatic Cell Degradation with Glass Beads				
• Protease (0.5 %)	24	11.1 ± 0.07^{ef}	5.4 ± 0.21^{f}	7.7 ± 0.99^{ab}
• Protease (1.0 %)	24	7.8 ± 0.31^{d}	$5.0 \pm 0.14^{e f}$	7.2 ± 0.83^{ab}
• Cellulase (0.5 %)	18	$11.4 \pm 0.15^{f g}$	5.4 ± 0.07^{f}	9.9 ± 0.57^{ab}
• Cellulase (1.0 %)	24	$10.9 \pm 0.30^{e f}$	4.9 ± 0.14^{e}	4.7 ± 6.51^{ab}
4) Dual-Enzyme Cell Degradation with Glass Beads				
a) Simultaneous Enzyme Addition:				
• pH 5.0	18	10.8 ± 0.12^{ef}	6.0 ± 0.21^{g}	2.2 ± 2.91^{ab}
• pH 6.5	24	11.3 ± 0.46^{fg}	5.0 ± 0.14^{ef}	4.9 ± 0.96^{ab}
b) Sequential Enzyme Addition:				
• CP	36	12.1 ± 0.03^{g}	6.6 ± 0.14^{h}	1.9 ± 0.51^{a}
• PC	36	12.2 ± 0.58^{g}	5.8 ± 0.00^{g}	1.8 ± 0.55^{a}

* Time refers to the duration required to achieve the highest protein concentration. Data are presented as mean ± standard deviation (SD) of duplicate experiments (n = 2).

**Different lowercase letters within the same column indicate statistically significant differences among treatments as determined by one-way ANOVA followed by Tukey's post hoc multiple comparison test ($p < 0.05$).

***Cell disruption percentage (%) was calculated as 100% − intact cells (%). For example, a value of 11.8% intact cells corresponds to 88.2% cell disruption.

Cellulase at 0.5% yielded the highest protein content (8.5%) without glass beads. Higher cellulase concentration (1.0%) did not significantly ($p>0.05$) increase protein yield. This suggests an optimal enzyme concentration exists, beyond which further increase might not be beneficial. This may be due to excessive degradation affecting protein integrity or increased

viscosity limiting enzyme activity (Uribe & Sampedro, 2003; Yao, 2025). Once combined with glass beads, protein content increased to 11.4%. Cellulase and protease treatment at an optimal concentration (e.g., 0.5%), significantly increased protein release compared to lower or higher concentrations (Table 1). This synergistic degradation indicates that the protease effectively digests outer wall-associated proteins and mannoproteins to unmask the complex cell wall matrix, while cellulase damages the structural polysaccharide components, particularly hydrolyzing the β-glucan framework to facilitate intracellular protein release (Jali et al., 2023; Kordialik-Bogacka, 2013). Beyond the optimal concentration, enzyme saturation or inhibition might occur, limiting further protein release.

Sequential addition of cellulase and protease with glass beads resulted in the highest protein extraction (12.2%) after 36 hours, significantly greater than either mechanical or enzymatic treatment alone (Table 1). Sequential combinations of cellulase and protease paired with glass beads resulted in the highest overall protein extraction (up to 12.2%) after 36 hours, achieving yields significantly greater than either standalone mechanical or single-enzyme treatments (Table 1). Statistically, the specific sequence of enzyme addition whether protease followed by cellulase (PC) or cellulase followed by protease (CP) did not yield a significant difference ($p>0.05$), indicating that both sequential approaches are highly effective at destabilizing the cell wall matrix. This dual efficacy suggests two equally viable synergistic pathways: a cellulase pre-treatment may fracture the rigid inner β-glucan architecture to expose underlying proteins, whereas an initial protease treatment degrades the outer wall-associated mannoprotein shield, thereby enhancing the accessibility of the inner structural polysaccharides to subsequent cellulase action. In both configurations, the initial enzymatic weakening of the complex *C. utilis* cell wall drastically reduces its mechanical integrity, making it far more susceptible to the subsequent crushing action and shear forces exerted by the glass beads.

The present findings are in agreement with previous studies demonstrating that the efficiency of intracellular component recovery is highly dependent on the disruption strategy employed. Bzducha-Wróbel et al. (2014) reported considerable differences in β-glucan recovery among mechanical, chemical and autolytic treatments, emphasizing the importance of selecting an appropriate cell disruption approach. Similarly, Jacob et al., (2019) observed that enzymatic and mechanical treatments differ substantially in their disruption efficiency and may complement one another when applied in combination.

More recently, Moyo et al. (2025) demonstrated that autolysis and enzyme proteolysis pretreatments enhanced subsequent mechanical rupture, thereby improving sustainable yeast protein recovery. Furthermore, Mantri et al. (2025) highlighted the advantages of integrated fungal cell disruption strategies, particularly the use of pretreatment techniques to weaken recalcitrant cell wall structures prior to mechanical processing. Consistent with these reports, the present study showed that sequential protease–cellulase treatment combined with glass bead disruption resulted in the highest protein recovery from *C. utilis* cultivated on pineapple waste substrate, suggesting that integrated disruption strategies have considerable potential for sustainable yeast extract production.

From an industrial perspective, although the 42h incubation period employed in the sequential enzymatic treatment may be considered relatively long for large-scale applications, the enhanced protein recovery achieved through enzymatic pretreatment followed by mechanical disruption may compensate for the extended processing time by reducing the energy consumption typically associated with intensive conventional bead milling. While enzyme cost remains an important consideration, the utilization of inexpensive pineapple waste as a

fermentation substrate may partially offset enzyme and operational expenses, thereby improving the economic feasibility of the process and supporting sustainable bioprocessing and circular bioeconomy initiatives.

Despite the promising results obtained in this study, several considerations should be addressed before industrial implementation. Although sequential enzymatic treatment enhanced protein recovery, the relatively long incubation period and the cost associated with commercial enzymes may pose challenges for large-scale applications (Middelberg, 1995; Moyo et al., 2025). Future studies could explore the feasibility of utilizing bromelain-rich pineapple processing residues as an alternative proteolytic enzyme source, which may further improve process economics and strengthen the integration of agro-industrial waste valorization within a circular bioeconomy framework.

In addition, while the combined enzymatic–mechanical approach demonstrated superior protein recovery, other physical disruption techniques, such as high-pressure homogenization and ultrasonication, have also been reported as effective methods for recovering intracellular products from microbial biomass, often providing shorter processing times and opportunities for continuous operation (Middelberg, 1995; Jacob et al., 2019; Mantri et al., 2025). Comparative techno-economic assessments among these technologies and the present approach would provide valuable insights into process scalability, operational feasibility, and commercial viability. Nevertheless, the findings of the present study establish a foundation for developing sustainable and integrated yeast protein extraction strategies utilizing inexpensive agro-industrial substrates.

Microscopic observations provided qualitative confirmation of the effectiveness of the different cell disruption methods applied. Light microscopy (Figure 1) revealed clear morphological differences between untreated and treated *C. utilis* cells. Untreated cells exhibited intact, well-defined cell walls with uniform shapes and internal structures, characteristic of viable yeast cells. In contrast, cells subjected to the combined enzymatic and mechanical disruption treatment showed visibly ruptured cell walls, cell shrinkage, and disrupted morphology. The release of intracellular contents into the surrounding medium further affirmed successful lysis.

Transmission electron microscopy (TEM) images (Figure 2) offered deeper insights into ultrastructural changes. Ultrastructural analysis was focused specifically on the combined method (0.5% cellulase and glass beads at pH 5.0 for 18 hours). Although the sequential enzyme applications achieved the highest absolute numerical protein yields, they were statistically equivalent ($p>0.05$) to this combined treatment. Therefore, due to its significantly shorter processing time and superior cost-effectiveness, the combined approach was chosen as the optimal operational process for detailed microscopic validation.

As shown in Figure 2, untreated *C. utilis* cells retained their dense, multilayered cell wall and intact cytoplasmic contents, indicating structural integrity. However, cells treated with this optimized combination of 0.5% cellulase and glass beads displayed significant cell wall thinning, fragmentation, and visible breaches in the membrane structure. The cytoplasmic content appeared diffused or absent, confirming extensive release of intracellular proteins. These visual observations align with the quantitative data, particularly the elevated protein yields and reduced intact cell percentages, validating the synergistic effect of enzymatic weakening paired with mechanical rupture.

Overall, both microscopic techniques complement the biochemical analyses, reinforcing the conclusion that sequential and integrated treatments effectively compromise the yeast cell wall to maximize protein extraction.

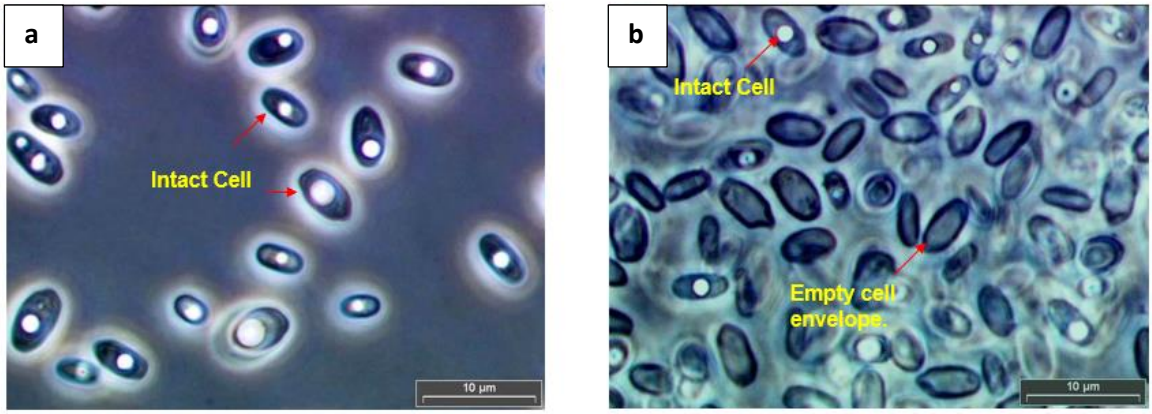


Figure 1 (a) Yeast cell before the degradation method; (b) Yeast cells after the degradation method

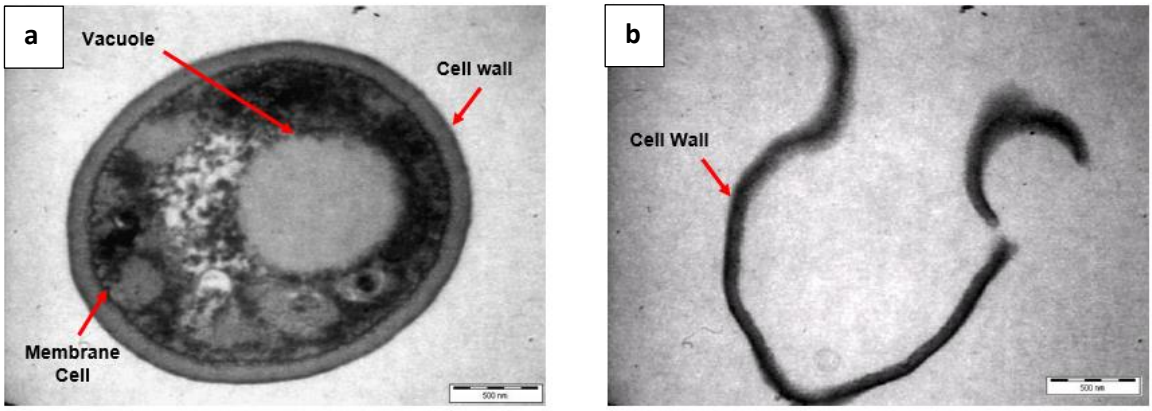


Figure 2 TEM images of *C. utilis* cells (A) before and (B) after degradation using the combined method (0.5% cellulase and glass beads at pH 5.0 for 18 hours)

CONCLUSION

This research confirms that integrated enzymatic and mechanical disruption strategies significantly enhance protein extraction from *C. utilis*. While sequential enzyme combinations (protease and cellulase) paired with glass bead agitation achieved the highest absolute numerical protein recovery of 12.2%, this yield was statistically equivalent ($p>0.05$) to the less intensive, combined single-enzyme method (0.5% cellulase with glass beads at 18 hours). Consequently, the combined approach was identified as the optimal process configuration, offering an efficient and potentially scalable industrial extraction strategy by significantly reducing processing time and secondary enzyme costs. These findings clearly demonstrate the synergistic effect of enzyme-mediated cell wall destabilization paired with mechanical shear forces. Furthermore, the successful utilization of pineapple waste as a growth medium highlights the potential for integrating agro-industrial waste valorization with bioprocess optimization. Overall, this study contributes to sustainable biotechnology by providing an improved extraction workflow that supports cost-efficient manufacturing and circular bioeconomy initiatives in yeast extract production.

ACKNOWLEDGEMENT

Authors are grateful to IRPA grant awarded by Ministry of Science, Technology and Innovation of Malaysia and Universiti Sains Malaysia for the financial assistances.

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