Asia-Pacific Journal of Science and Technology: Volume: 30. Issue: 03. Article ID.: APST-30-03-13. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by Research Department,
Khon Kaen University, Thailand

Effect of culture conditions on chitin recovery from shrimp heads by the oleaginous yeast *Yarrowia lipolytica*

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Received 23 October 2023
Revised 15 October 2024
Accepted 26 November 2024

Abstract

Yarrowia lipolytica is an oleaginous yeast strain proficient in producing extracellular protease and acid, and it has demonstrated the capacity to recover chitin from shrimp by-products. This study examined the submerged fermentation parameters, including incubation time, pH, and inoculum size, utilizing *Y. lipolytica* to extract chitin from whiteleg shrimp heads. The fermentation efficacy was evaluated through deproteinization and demineralization levels. The results indicated that *Y. lipolytica* had a notable capacity for synthesizing extracellular protease enzymes, although its potential to acidify the shrimp head environment is minimal. An increment of the deproteinization level with the incubation time, in parallel with the protease activity of the culture, was observed. The suitable pH for fermentation was determined to be 6.0. An inoculum size between 6 and 8 log CFU/mL showed an unclear effect on fermentation efficacy. The deproteinization level reached 91.61±0.54%, while the demineralization level was moderate at 47.17±1.71%.

Keywords: By-product upgradation, Chitin recovery, Shrimp head, *Yarrowia lipolytica*, Yeast fermentation

1. Introduction

The rapid development of the shrimp farming and processing industry has resulted in an increase in the amount of shrimp by-products released into the environment. Over 45–60% of the whole shrimp returns to waste or by-products [1]. Moreover, shrimp by-products are a valuable and potential source of raw materials. Shrimp heads and shells contain plural bioactive substances such as protein, astaxanthin, minerals, and chitin that can be exploited and utilized to produce products of high economic value in the pharmaceutical, functional foods, human food, animal feed, fertilizer, and other industries [2]. Shrimp heads account for the majority of shrimp by-products as they constitute about 33.63–53.09% of the whole shrimp weight [3]. The head of whiteleg shrimp, *Litopenaeus vannamei* contains 17.6±1.1% chitin, making it a good resource for chitin recovery [4]. The recovered chitin can be bleached or transformed into chitosan or chitooligosaccharides and may have applications in wastewater treatment, food, biomedicine, agriculture, textile and paper, biotechnology, or even cosmetics [5].

Chitin in crustacean skeletons is firmly integrated with protein and interlaced with calcium salts, resulting in a robust and durable structure. Consequently, the extraction of chitin requires the elimination of protein and calcium salt. Protein may be extracted from the chitin-associated network by hydrolysis using alkali or proteolytic enzymes, while calcium salts can be solubilized by acids (either inorganic or organic) or removed when the chitin-protein matrix is disrupted. This can be accomplished by chemical or biological methods [5]. The microbial method was reported as more effective for chitin recovery than chemical methods as it can prevent chitin alteration from harsh chemical conditions [6]. Typical fermentation employs lactic acid bacteria or protease-producing bacteria with the addition of glucose as carbon source supplement. The efficacy of chitin recovery by microbial fermentation was believed to be related to cellular protease activities and cellular acid production [7, 8]. Factors

such as inoculum size, culture pH, and culture incubation time are shown to influence the efficiency of the process [7].

Yarrowia lipolytica is a biological model with a full range of biotechnology applications [9]. Yeast was reported as a biological agent used to synthesize aromatic compounds, organic acids, and enzymes, especially extracellular proteases [10]. A recent study showed that shrimp head fermentation with *Y. lipolytica* resulted in higher efficacy in chitin recovery than bacterial fermentation or enzymatic hydrolysis. Two days of fermentation with yeast resulted in 80.9% deproteinization, while this figure was only 65.4% for *Bacillus subtilis* fermentation and only 76.9% for enzymatic hydrolysis [4]. Demineralization obtained with yeast fermentation was also 2.9–3.3 fold greater than with bacterial fermentation or enzymatic hydrolyzation [4].

This paper investigated fermentation parameters, including incubation time, pH, and inoculum size, when culturing the oleaginous yeast *Y. lipolytica* VTCC0544 on shrimp head medium, evaluating the impact of these conditions on demineralization and deproteinization efficiency, along with the relationship between extracellular protease and acid production during yeast growth on shrimp head medium and chitin recovery efficiency.

2. Materials and methods

2.1 Materials and yeast strain

Whiteleg shrimp (*L. vannamei*) head was collected at a seafood factory in Khanh Hoa province, Vietnam. For the experiments, the head was homogenized with a screw extruder (3 mm), divided into 100 g Polyethylene (PE) bags, and stored at –20 °C.

The yeast *Y. lipolytica* VTCC0544 was stored in 15% glycerol at –20 °C and activated on yeast extract peptone dextrose agar plates (YPDA) (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, agar 15 g/L) for 48 h at 29±1 °C before use. Preculture was prepared with five loops inoculated in 100 mL yeast extract peptone dextrose (YPD) (YPDA without agar) medium for 24 h with shaking at 250 rpm at 29±1 °C. The biomass was harvested in sterile physiological water by centrifugation (1600 *g*, 5 min) and used for inoculation.

2.2 Biochemical assay

2.2.1 Extracellular protease assay

Skim milk plates (400 mL skim milk, 600 mL distilled water, 15 g agar) were used to detect extracellular protease production [11]. Yeast was inoculated on skim milk plates and incubated at 29±1°C for 7 days. The radii of halos (mm) and yeast colonies (mm) were measured every day. The radius of the hydrolyzed zone was calculated by subtracting the radius of the colony from that of the halo.

2.2.2 Extracellular acid production assay

Organic acid production was tested using YPDA and yeast extract peptone agar (YPA) (YPDA without glucose) media supplemented with the pH indicator bromocresol green (0.1 g/L) [12]. The initial pH of the medium was adjusted to 5.6 with 5 N KOH solution. Yeast were inoculated on agar plates and incubated at 29±1°C for 7 days. A yellow zone was detected around yeast colonies corresponding to acid secretion. The radii of the yellow zone (mm) and yeast colony (mm) were measured every day. The radius of the acidified zone was calculated by subtracting the radius of the colony from the radius of the yellow zone.

2.2.3 Protease activity measurement

Ten milliliters of culture was centrifuged (1600 *g*, 5 min) to eliminate the residue. The supernatant was collected to determine the protease activity using the Anson method [13].

2.2.4 Determination of yeast cell density

One milliliter of culture was collected and suitably diluted. The yeast cell density (cells/mL) was measured using a Mallassez counting chamber [14].

2.2.5 Culture pH measurement

Ten milliliters of culture was centrifuged (1600 *g*, 5 min) to eliminate the residue. The supernatant was collected to determine the pH using a pH meter (Hanna instruments HI2211).

2.3 Preparation of shrimp head medium

Shrimp heads were thawed at room temperature before use for fermentation. Ten grams of thawed shrimp heads were autoclaved with 20 mL of distilled water in a 100-mL Erlenmeyer flask and inoculated with yeast biomass to attain the desired inoculum size. The fermentation was performed by shaking at 250 rpm and 29±1 °C. To investigate the impact of pH on fermentation efficacy, 15 mM phosphate buffer at different pH levels was used instead of distilled water to prepare shrimp head medium.

2.4 Determination of fermentation efficacy

The protein content was determined by the Kjeldahl method with a coefficient of 6.25. The ash content was determined by heating in a furnace at 550 °C. The water content was determined by drying to a constant weight in an oven at 105 °C and used for calculation on a dry basis [4]. These analyses were used to determine the fermentation efficacy through the deproteinization and demineralization levels.

2.4.1 Determination of the deproteinization level

The deproteinization level was calculated using the following Equation (1):

$$Deproteinization\ (\%) = \frac{P_S \times S - P_R \times R}{P_S \times S} \times 100 \quad (1)$$

In which P_S and P_R were the protein content of the original sample and residue sample after fermentation, respectively, and S and R were the weight of the original sample and residue sample, respectively, on a dry basis [15].

2.4.2 Determination of the demineralization level

The demineralization level was calculated using the following Equation (2):

$$Demineralization\ (\%) = \frac{A_S \times S - A_R \times R}{A_S \times S} \times 100 \quad (2)$$

In which A_S and A_R were the ash content of the original sample and residue sample, respectively, after fermentation, and S and R were the weight of the original sample and residue sample, respectively, on a dry basis [15].

2.5 Statistical analysis

Three replicates of each experiment were performed. Data were analyzed with Statistica 7.1 (StatSoft, France). ANOVA was performed using Duncan's multi-range test with *p* values less than 0.05. Correlation analysis was performed using Pearson's correlation.

3. Results and discussion

3.1 Biochemical properties of Y. lipolytica VTCC0544

3.1.1 Extracellular protease production

The proteolytic capacity of *Y. lipolytica* VTCC0544 was analyzed using skim milk agar plates for its ability to hydrolyze skim milk and to create a clear zone around the colony (Figure 1).

The results show that the radius of the halo increased in parallel with that of the colony in the first 6 days and continued to increase when the colony ceased to grow, resulting in a continuous increase in the radius of hydrolyzed zone (Table 1). The yeast could synthesize proteases to hydrolyze protein to meet cellular nitrogen requirements during growth. When *Y. lipolytica* was cultivated in peptone or protein-rich media, the gene *XPR2* encoding alkaline extracellular protease (AEP) was activated, leading to AEP production of 1–2 g/L [10]. *Y. lipolytica* has been reported to produce extracellular proteases, which were able to hydrolyze the casein in skim milk to produce 300–350 mg leucine/100 mL skim milk [16]. *Y. lipolytica* was also reported as the best yeast strain regarding proteolytic activity among strains isolated from cheese [17]. This demonstrates a very promising capability for shrimp head protein hydrolysis.

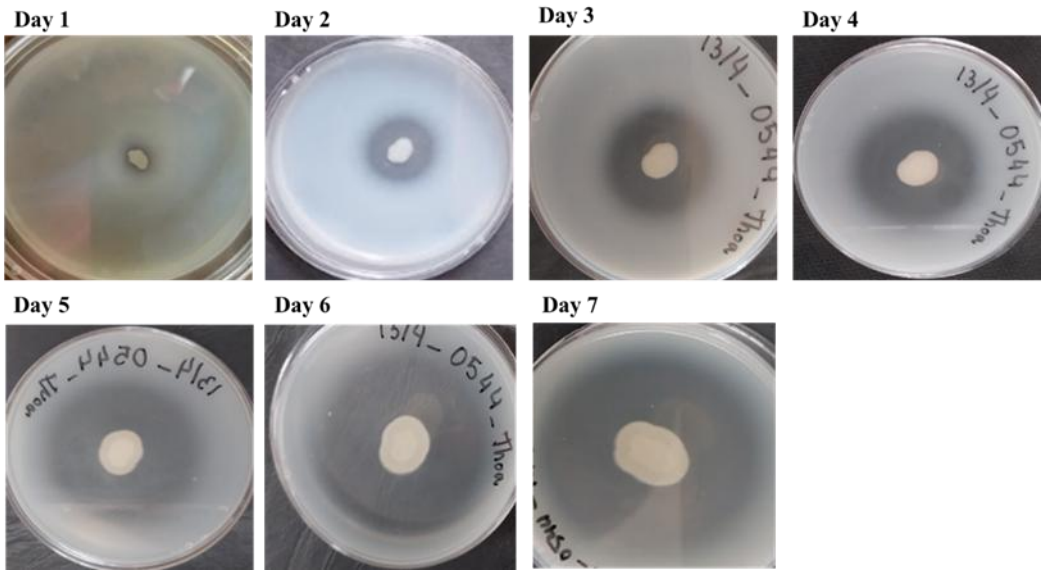


Figure 1 Protease assay on skim milk plate of *Y. lipolytica* VTCC0544.

3.1.2 Extracellular acid production

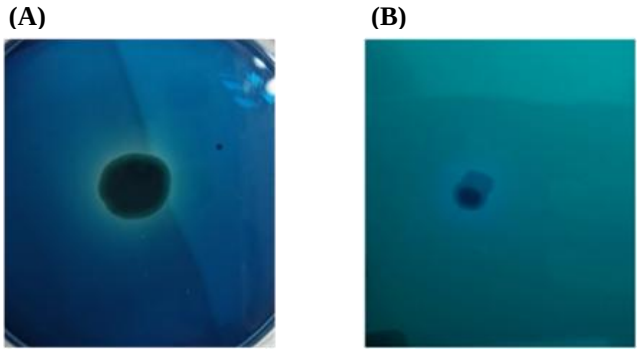
Extracellular acid production by *Y. lipolytica* on peptone medium is shown in Table 1 and Figure 2. Our results show that *Y. lipolytica* can accumulate acid when cultured on YPDA, which is peptone medium with glucose, as a yellow zone was observed around the yeast colony (Figure 2A). The productivity was low where the radius of the acidified zone was lower than 1.5±0.0 mm (Table 1). In the case of YPA medium, no acidification was observed as the pH indicator did not change to yellow but to blue (Figure 2B), which corresponded to a pH increase [12]. This indicates that the yeast did not synthetize extracellular acid on peptone medium in the absence of glucose.

Y. lipolytica can biosynthesize and secrete different organic acids, such as isocitric acid, succinic acid, α-ketoglutaric acid, itaconic acid and the most prominent, citric acid [18]. Since citric acid accumulation starts after all available nitrogen has been consumed, the main requirement for citric acid production by this yeast is a nitrogen content deficit in the culture medium [19]. This is not the case with yeast extract peptone (YP) medium nor shrimp head medium. This fact may restrict the shrimp head demineralization capacity of the yeast.

Table 1 Protease and acid production on agar plates of *Y. lipolytica* VTCC0544.

	Incubation time (days)						
	1	2	3	4	5	6	7
Radius of hydrolyzed zone (mm)	1.0±0.0^{a}	5.0±0.0^{b}	5.2±0.3^{b}	7.0±0.0^{c}	10.2±0.3^{d}	13.8±0.8^{e}	17.7±1.0^{f}
Radius of acidified zone (mm)	0.0±0.0^{a}	0.2±0.1^{a}	0.4±0.1^{b}	1.4±0.1^{d}	1.5±0.0^{d}	1.1±0.1^{c}	0.5±0.0^{b}

[*] letters indicate a significant difference with $p = 0.05$ (within the same row)
[**] protease production was determined on skim milk plates, and acid production was determined on YPDA plates



[*] 4 days of incubation

Figure 2 Acid production assay of *Y. lipolytica* VTCC0544 when cultured on a peptone plate: (A) YPDA medium and (B) YPA medium.

3.2 Shrimp heads fermentation using Y. lipolytica VTCC0544

3.2.1 Effect of incubation time

The effect of incubation time on shrimp head fermentation was investigated for 7 days, and the results are shown in Table 2. A similar relationship between deproteinization and demineralization efficiency was observed. Both parameters increased sharply after the first days of fermentation and reached the plate after 5 days of fermentation. The deproteinization level was already 79.95 ± 0.93% after 1 day of fermentation and peaked at 85.71 ± 1.08% after 5 days of fermentation. Regarding demineralization efficacy, it was 35.36±0.1% after 1 day of fermentation and then reached 44.50±0.89% after 5 days of fermentation. These results align with those obtained by fermenting shrimp heads using indigenous yeast strains isolated from traditionally fermented food products. The deproteinization level under suboptimal conditions ranged from 60% to 80%, whereas the demineralization level exhibited significant variability, ranging from around 2% to 50% [20].

The cell density, pH, and extracellular protease activity can influence the effectiveness of mineral and protein reduction. The pH of shrimp heads medium was 8.8±0.1 and decreased to 7.6±0.0 after 1 day of fermentation. Besides, the proteins in shrimp heads were reported to exist in the form of calcium-protein-chitin complexes, which are localized in the inner and intermediate membranes of shrimp by-products [2]. As a result, in the early stages of fermentation, the yeasts were in a nitrogen-starved state, which led to adenosine monophosphate (AMP) deaminase activation. This enzyme catalyzed AMP into inosine monophosphate and ammonia, reducing the cofactor required for ATP-citrate lyase, leading to disruption of the Krebs cycle and the accumulation of metabolic products such as citric acid in the early stages of the cycle. The citric acid in the mitochondria then exits the cytoplasm by exchange with oxaloacetate in the cytoplasm before secretion into the broth via the specific transporter Yhm2p [21], resulting in a decrement in the pH of the culture. This effect could contribute to the demineralization of the shrimp heads by solubilization. The variation in the efficacy of proteinization and demineralization could also depend on the protease activity produced by microorganisms, as well as the protease's mobility to the substrate and microbial growth. The protease activity of the 1-day culture increased by 42.96%, while the cell density increased by 1.26 log/mL (Table 2). These results explain the sharp increment in deproteinization and demineralization efficacy during the first day of fermentation.

During the subsequent days of fermentation, the yeast entered the end of the log phase and approached the stationary phase on the fifth day. Besides, the protease activity continued to increase, reaching the highest value of 10.44±0.47 AU/mL after 4 days of fermentation and then decreased gradually (Table 2). This may have been due to the decrease in protein demand when the culture reached the stationary phase. The level of deproteinization increased with the protease activity and stabilized after the last protease reached maximum activity. Otherwise, the culture's pH increased gradually from the second day of fermentation, reaching 8.55±0.34 on the seventh day (Table 2). The proteolysis in the culture of *Yarrowia* sp. was reportedly associated with media alkalization, which resulted in an increment of the culture pH to 8.5–9.0 [22]. This increment in pH may explain the cessation of demineralization of shrimp heads.

Table 2 Effect of incubation time on shrimp heads fermentation by yeast *Y. lipolityca*.

	Incubation time (days)							
	0	1	2	3	4	5	6	7
Cell density (log CFU/ mL)	6.34±0.05^{a}	7.6±0.04^{b}	7.82±0.06^{c}	7.9±0.1^{c}	8.01±0.08^{d}	8.05±0.06^{de}	8.1±0.06^{de}	8.14±0.04^{e}
Culture pH	8.76±0.08^{f}	7.62±0.04^{a}	7.79±0.34^{ab}	7.92±0.26^{ab}	8.03±0.27^{abc}	8.16±0.16^{bcd}	8.4±0.29^{def}	8.55±0.34^{ef}
Protease activity (AU/mL)	5.47±0.24^{a}	7.82±0.34^{c}	8.87±0.27^{d}	10.06±0.34^{e}	10.44±0.47^{f}	9.54±0.47^{e}	7.35±0.34^{c}	6.25±0.2^{b}
Deproteinization (%)	nd	79.95±0.93^{a}	82.04±0.58^{b}	84.64±0.08^{c}	85.59±1.06^{cd}	85.71±1.08^{cd}	86.54±1.21^{d}	86.42±1.23^{cd}
Demineralization (%)	nd	35.36±0.1^{a}	42.01±0.37^{b}	44.41±1.09^{c}	44.28±1.69^{c}	44.46±0.89^{c}	45.96±0.7^{cd}	46.87±1.25^{d}

* letters indicate a significant difference with $p = 0.05$ (within the same row)
nd = non determination
culture condition: inoculum size 6 log, pH natural (~8.8)

3.2.2 Effect of initial pH

The effect of the medium's initial pH on shrimp heads fermentation was investigated by adjusting the medium pH to 5.0, 6.0, and 7.0 (Table 3). The results show that a weak acid medium could be more favorable to both deproteinization and demineralization than a neutral or alkaline medium. The deproteinization efficiencies were 87.50±0.99% and 88.43±0.54% for pH 5.0 and 6.0, respectively, which were higher than 85.19–85.71% for pH ≥ 7.0. This difference in the deproteinization level corresponded to the difference in the protease activity of the culture (Table 3), which was decreased when the culture was varied from acid to alkaline pH. *Y. lipolytica* could synthesize two groups of extracellular proteases, including acid extracellular protease (AXP) and AEP. AXP was

produced in culture over the range pH 2–6 and AEP at pH 6–9 [23]. AEP expresses similar enzyme activity over the range pH 5.0–7.5, while AXP activity was reported as acid dependent, and no enzyme activity was detected for the culture at pH from 6.5 [24]. The highest protease activity of the culture was recorded at pH 6.0. Besides, pH 6.0 was also the most suitable pH for yeast growth as the cell density was the highest (8.27±0.12 log CFU/mL). The dense population could require more nitrogen sources to satisfy cell growth.

A similar tendency was observed with demineralization. The highest demineralization level was obtained at pH 6.0, which was 45.62±0.41% and was 1.18–1.31 fold higher than the demineralization level obtained at two other pHs. The correlation analysis results show a high correlation between the demineralization and deproteinization levels with r = 0.82 (p = 0.007). One possible explanation is that during protein hydrolysis, the calcified protein-chitin complex is broken, and calcium is released [25].

Table 3 Effect of initial pH on shrimp heads fermentation by yeast *Y. lipolityca*.

	Initial pH			
	5.0	6.0	7.0	Natural
Cell density (log/ mL)	8.13±0.15^{b}	8.27±0.12^{b}	7.77±0.24^{a}	8.05±0.06^{ab}
Protease activity (AU/mL)	11.54±0.5^{a}	12.33±0.57^{a}	10.23±0.31^{a}	9.54±0.47^{a}
Deproteinization (%)	87.5±0.99^{b}	88.43±0.54^{b}	85.19±0.88^{a}	85.71 ± 1.08^{a}
Demineralization (%)	38.75 ± 1.02^{b}	45.62±0.41^{c}	34.85±0.52^{a}	44.46±0.89^{c}

* letters indicate a significant difference with p = 0.05 (within the same row)
** the natural pH of shrimp heads medium was ~8.8
***culture condition: inoculum size 6 log, incubation time 5 days

3.2.3 Effect of inoculum size

The effect of the inoculum size on shrimp heads fermentation efficacy is shown in Table 4. The typical inoculum size ranges from 5–20% v/v or 3–8 log CFU/mL [7]. In this study, when the inoculum size was increased from 6 log CFU/mL to 8 log CFU/mL, the deproteinization increased by only 3.59%, while there was no significant difference in the demineralization level. Despite the different inoculation levels, the cell density attained a similar range of 8.0–8.2 log CFU/mL after 5 days where the growth reached the stationary phase (as shown in the Table 4). The pH of the cultures was similar in range, 7.98–8.23, which may explain the same degree of demineralization. Deproteinization is thought to be related to microbial protease activity. This could also be correlated to the enzyme accessibility as the calcium in a calcified protein-chitin complex may prevent enzymes from penetrating cells to break down protein. Therefore, even though the protease activity in the culture broth increased with the inoculum size, the deproteinization level was not clearly ameliorated. Similar results were observed when using *Bacillus* bacteria to extract chitin from shrimp shells [25].

Table 4 Effect of inoculum size on shrimp heads fermentation by yeast *Y. lipolityca*.

	Inoculum size (log/mL)		
	6	7	8
Cell density (log/mL)	8.27±0.12^{b}	8.09±0.01^{a}	8.02±0.02^{a}
5-day culture's pH	8.23±0.08^{a}	8.13±0.07^{a}	7.98±0.19^{a}
Protease activity (AU/mL)	12.13±0.57^{a}	13.38±0.48^{a}	15.39±0.29^{b}
Deproteinization (%)	88.43±0.54^{a}	90.63±2.08^{ab}	91.61±0.54^{b}
Demineralization (%)	45.62±0.41^{a}	44.81±2.15^{a}	47.17±1.71^{a}

* letters indicate a significant difference with p = 0.05 (within the same row)
**culture condition: pH 6.0, incubation time 5 days

4. Conclusion

The oleaginous yeast *Y. lipolytica* VTCC0544 was demonstrated as a potential proteolytic agent for chitin recovery from shrimp heads with the ability to attain deproteinization and demineralization levels of 91.61% and 47.17%, respectively. The fermentation efficacy was improved with increasing incubation time and inoculum size. The culture pH also had impacts on fermentation, and a weak acid pH could favor the deproteinization and demineralization level. These results show the feasibility of using yeast to recover chitin from shrimp by-products, in addition to fermentation utilizing other microorganisms. Besides chitin, this clean method may yield several additional valuable compounds, including proteins, pigments, and minerals. This is a prospective approach that requires investigation to enhance the manufacturing scale.

5. Acknowledgment

We acknowledge the support of time and facilities from Ho Chi Minh City University of Technology (HCMUT), VNU-HCM for this study

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