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Research Article

Deproteinization and demineralization of shrimp head using yeasts isolated from Nemchua

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ABSTRACT
In recent years, the utilization of seafood processing by-products has attracted considerable attention due to their potential as a source of natural biopolymers. Shrimp processing by-products, particularly head material, are recognized as a rich source of chitin - a valuable natural polysaccharide widely used in various industrial and biomedical applications. This study investigated the isolation of indigenous yeasts from Nemchua, a traditional Vietnamese fermented meat product, and evaluated their capacity for chitin recovery from whiteleg shrimp (*Litopenaeus vannamei*) heads. Eleven yeast strains were successfully isolated and purified from Nemchua samples. Their morphological characteristics and representative carbohydrates utilization profiles were also evaluated. Preliminary screening demonstrated that all isolates exhibited culturability on shrimp heads, indicating their potential to utilize shrimp processing waste as a nutrient source. Fermentation was proceeded under conditions of 5 days at an initial pH of 8.87, with an inoculum of 10^8 CFU/g, and fermentation efficiency was evaluated based on deproteinization, demineralization, and crude chitin recovery. The results showed that, all strains demonstrated deproteinization level exceeding 60%, with highest reaching 80.5% observed in the most potent strains. Demineralization levels varied significantly, ranging from 1.9 to 50.1%, while the pH of fermented shrimp culture declined slightly from 8.87 to 8.32-8.80. Crude chitin recovery was also determined in the range of 38.1-64.8%. Deproteinization showed a significant negative correlation with crude chitin recovery ($r = -0.74$, $p < 0.001$), while no clear relationship was observed between demineralization and pH changes, suggesting that mineral removal may be associated with protein removal during fermentation rather than acidification alone. Among the isolates, strains N22 and N23 showed the most potential for industrial application, exhibiting high levels of deproteinization, demineralization and performance in chitin recovery. These findings highlight the potential of using indigenous yeast fermentation as a sustainable and green approach for the valorization of shrimp processing by-products.
Key words: Chitin recovery, yeast isolation, yeast fermentation, shrim head, nemchua

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INTRODUCTION

The fast growth of the shrimp farming and processing business has resulted in an increasing amount of shrimp byproducts being released into the environment. A considerable part of entire shrimp, roughly 45-60%, is discarded as waste or byproducts [1]. Shrimp byproducts are a valuable and promising source of raw materials. Shrimp heads and shells contain numerous physiologically active substances like as protein, astaxanthin, minerals, and chitin, which can be extracted and used to produce high-value products in the sectors of pharmaceuticals, functional foods, human food, animal feed, fertilizer …[2]. Shrimp heads comprise the majority of shrimp by-products, accounting for 33.63-53.09% of total shrimp weight [3]. The white shrimp head (*Litopenaeus vannamei*) contains $17.6 \pm 1.1\%$ chitin by weight, making it a potential source for chitin production [4].

Protein and chitin in shrimp tissue combine to form a protein-chitin matrix, which is subsequently heavily calcified to make a hard shell. As a result, the chitin recovery process contains two steps: one for protein removal (deproteinization) and another for calcium removal (demineralization). This can be accomplished using chemical or biological techniques. The microbial method has been shown to be more efficient in chitin recovery than the chemical method because it can prevent chitin transformation caused by the severe conditions of the chemical reaction [5].

There are two microbial fermentation strategies for recovering chitin from crustacean byproducts: lactic fermentation and non-lactic fermentation. The first approach relies on lactic bacteria to make lactic acid, which dissolves calcium carbonate in crustacean shell and releases the softer protein-chitin layer. Many publications have reported excellent chitin recovery

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from shrimp byproducts using lactic bacteria like *Lactobacillus* spp.[6,7] or *Pediococcus* spp.[8,9].

The non-lactic fermentation process relies on the proteolytic capacity of microorganism. The by-products are employed as a nitrogen source for microbial growth, lowering the pH of the fermentation medium and so improving the demineralization process of shrimp by-products. Some protease-producing bacteria, such as *Bacillus licheniformis*, *B. amyloliquefaciens*, and *B. subtilis*, are utilized in shrimp shell fermentation to achieve substantial protein reduction (88.3-95.6%) and moderate demineralisation (37.1–67.2%)[10].

Recent studies have shown that beside proteolytic bacteria, yeast has the potential to recover chitin from shrimp heads. Some yeast species are also capable of producing extracellular proteases. Fernández et al.[11] found that 53 out of 141 wild yeast isolates were able to hydrolyze casein, identifying them as *Metschnikowia pulcherrima* and *Pichia membranifaciens*. Extracellular protease from yeast capable of hydrolyzing protein from shrimp waste into liquor with soluble protein hydrolysates, peptides, free amino acids and release protein-related minerals which lead to deproteinization and demineralization[2]. Yeasts isolated from indigenous sources, while not particularly efficient in by-product treatment, desserve more investigation[4].

Nemchua, a traditional Vietnamese fermented meat product, exhibits a yeast density ranging from 10^5-10^6 CFU/g. In a study of fermented pork roll products from Hanoi, Thanh Hoa, and Vinh, researchers isolated 84 yeast strains, with *Candida* sp., *Pichia* sp., *Debaryomyces hansenii*, and *Trichosporon beigelii* being the predominant species[12]. This highlights Nemchua's potential as a valuable source for indigenous yeast strains capable of fermenting and processing food by-products.

Objective of this work is to isolate yeast strains from nemchua to investigate their capacity to deproteinize and demineralize on shrimp heads. The study aims to evaluate the potential of utilizing indigenous microbial methods for chitin recovery from shrimp head waste.

MATERIALS AND METHODS

Materials

Whiteleg shrimp (size 30) were purchased in Ba Ria - Vung Tau, Vietnam. The shrimp must be alive, healthy, and intact. After harvested, the shrimp was transported in ice in Styrofoam boxes to laboratory in Ho Chi Minh City; Shrimp were washed, decapitated, and separated into zip bags before being frozen at -20 °C. Before the experiment, shrimp heads were pressed with a screw press to obtain the solid fraction. Nemchua samples were collected in Tho Xuan, Thanh Hoa, transported to laboratory in sterilized PE bag and stored at 5-10 °C for yeast isolation.

Isolation of yeasts from nemchua samples and physiological properties

Yeast isolation was effectuated as described[4]. Approximately 10 g of the sample was homogenized in 10 ml sterile physiological saline solution and 1 ml of suspension was spreaded over a yeast isolation medium (YPD supplemented with 100 mg/L chloramphenicol). The plates were incubated at 30 ± 2 °C for 2-4 days. Individual colonies are evaluated for colony and cell morphology before being streaked 2-3 times into YPD medium to obtain purified colonies. The resulting yeast strains were encoded and stored in 15% glycerol at -20 °C. Yeast strains were activated on YPDA (48 h, 30-32 °C) before used.

The colony morphology of all strains was observed after culturing on YM plate (peptone 5 g/L, yeast extract 3 g/L, malt extract 3 g/L, glucose 10 g/L, agar 15 g/L) for 2 days, at 30 °C[13]. For physiological test, the strains were assessed for their ability to catabolite and ferment carbohydrates including glucose, sucrose, and lactose. The microorganisms were inoculated into test tubes containing 10 mL of M10 medium (carbohydrate 5 g/L, peptone 10 g/L, yeast extract 5 g/L with bromothymol blue as an indicator) and inverted Durham tube. A loop microorganism was innoculated and incubated under both aerobic and anaerobic conditions at 30 °C for 2 days and, observed for the acid production and gas formation[14].

Shrimp fermentation

Ten g of defrozen and pressed shrimp head was introduced into a 250 ml erlenmeyer with 10 ml distilled water. The mixture was autoclaved at 121 °C for 15 min. The pH of shrimp head medium was determined to be 8.87 ± 0.01.

Five loops of activated yeasts biomass were inoculated into 150 ml YPD for preculture (24 h, 30-32 ^{o}C). The biomass was then centrifuged (3500 rpm, 10 min), washed with phosphate buffer 0.2 M at pH 5.6, and inoculated into shrimp medium with an inoculum size of 10^8 CFU/ g shrimp head. The fermentation was conducted for 5 days. The crude chitin was collected using a 140 mesh sieve, washed with distilled water and used for chemical analysis[4].

Chemical analysis methods

Proximate composition analysis

The proximate composition was analyzed following Vietnamese standard methods. Water content was determined by drying to constant in an oven at 105 °C. Crude lipid content was determined by the Soxhlet method with diethyl ether extraction. Crude protein content was determined by the Kjeldahl method with a coefficient of 6.25. Ash content was determined by heating in a furnace at 550 °C.

Determination of chitin content in shrimp head

Chitin content was determined as described by Pham et al.[15]. Briefly, 2 g of dried shrimp head was immersed in 50 mL HCl 1 M (80 °C, 1 h) for demineralization, following by deproteinization in 100 mL NaOH 1 M (90 °C, 1 h). The resulting solid was filtered, washed twice with 15 mL acetone and dried to constant at 110°C. The chitin content (CT) was calculated using the following equation.

$$CT\ (\%) = \frac{m_2 \times 100}{m_1 \times \frac{100-MC}{100}}$$

in which m_1, m_2 are weight of sample before and after treatment, MC is the moisture content of the initial sample.

Determination of deproteinization, demineralization and crude chitin recovery

The efficacy of fermentation was determined through degree of deproteinization (DP), demineralization (DA) and crude chitin recovery (CCR), using the following calculations:

$$DP = \frac{P_{original} \times m_{original} - P_{residue} \times m_{residue}}{P_{original} \times m_{original}} \times 100$$

$$DA = \frac{A_{original} \times m_{original} - A_{residue} \times m_{residue}}{A_{original} \times m_{original}} \times 100$$

$$CCR = \frac{m_{residue}}{m_{original}} \times 100$$

in which $P_{original}$, $P_{residue}$ were protein content and $A_{original}$, $A_{residue}$were ash content of original sample and residue sample after treatment. $m_{original}$ and $m_{residue}$were weight of original and residue sample in dried weight basis, respectively[4].

Statistical analysis

All experiments were performed in triplicate. The difference was calculated using Statistica VII software's analysis of variance (ANOVA) function. A p value of less than 0.05 was recognized as a significant difference. The correlation between deprotinization and chitin recovery was conducted by Pearson correlation analysis, using Microsoft excel.

RESULTS AND DISCUSSION

Chemical composition of pressed shrimp head

The chemical composition of pressed shrimp head was shown in the Table 1. Similar to other research[4], the protein and ash were the principal component in the shrimp head, which include upto 76.5% of the dried matter. The resistant structure of the crustacean shell is formed by the reaction between protein and mineral, typically calcium carbonate[16]. Therefore, in order to liberate chitin, it is imperative to eliminate these proteins and minerals.

Physiological properties of yeasts isolated from nemchua

From the collected nemchua samples, 11 strains of yeast were isolated and purified. The majority of these strains display a spherical to oval morphology, with the exception of strains N12 and N13, which exhibit elongated cells. All strains showed typical morphological characteristics of yeast colonies: ivory white and convex colony with round or unround shape, smooth or rough surface (Table 2).

All the isolated strains could catabolite and ferment glucose. Only strain N41 could not assimilate saccharose. Beside, most of strains except N13, N24 and N32 could not use lactose as carbon source (Table 3).

The efficacy of fermentation is dependent upon several parameters, such as the origin and type of carbon sources[17]. Adding carbohydrates such as glucose[10], sucrose[18], and lactose[19] as carbon sources to the fermentation environment can enhance the deproteinization and demineralization effect. However, supplementing these carbon sources is often considered costly. Instead, recent studies have focused on using waste materials containing these carbohydrates as an alternative[20,21].

Deproteinization, demineralization and chitin recovery efficiency

Eleven isolated yeast strains were tested for fermentation of shrimp heads to recover chitin. Cultures were conducted without any supplemented nutrients and

Table 1: Chemical composition of pressed shrimp head

	Proximate content (%)				
	Water	Protein *	Lipid *	Ash *	Chitin *
Pressed shrimp head	68.2 ± 1	56.7 ± 0.1	8.0 ± 0.5	19.8 ± 0.3	13.6 ± 0.6

*Content calculated as percent of dried weight
Source: Authors. Data are presented as Mean ± SD (n=3)

Table 2: Colony and cells morphology of isolated yeasts

Strain	Colony's morphology	Cell morphology
N11	Irregularly round shape, ivory white, convex appearance, serrated edges, smooth and shiny surface, with diameter about 2 mm.	Spherical
N12	Irregularly round shape, ivory white, convex appearance, serrated edges, rough but shiny surface, with diameter about 2 mm.	Oval or allongated
N13	Irregularly round shape, ivory white, convex appearance, serrated edges, rough but shiny surface, with diameter about 2 mm.	Spherical or allongated
N21	Round shape, are ivory white, and have a convex appearance, continous edges, smooth and shiny surface, with diameter about 2 mm.	Oval
N22	Unround shape, ivory white, convex appearance, serated edges, smooth and shiny surface, with diameter about 1.5 mm.	Oval
N23	Round shape, ivory white, convex appearance, serrated edges, rough but shiny surface, with diameter about 2 mm.	Oval
N24	Round shape, ivory white, convex appearance, serrated edges, rough but dried surface, with diameter about 2 mm.	Spherical or oval
N31	Unround shape, ivory white, convex appearance, serated edges, rough but dried surface, with diameter about 2 mm.	Oval
N32	Round shape, ivory white, convex appearance, continous edges, rough but shiny surface, with diameter about 2 mm.	Oval
N41	Irregular unround shape, ivory white, convex appearance, serrated edges, smooth surface, with diameter about 2 mm.	Spherical
N42	Round shape, ivory white, convex appearance, serrated edges, smooth and shiny surface, with diameter about 2 mm.	Oval

Source: Authors. Cells were cultivated on YM plate, 48-72 h at 30-32 ^{o}C

the deproteinization, demineralization, chitin recovery efficiency and the pH of fermented shrimp heads were determined (Table 4).

As shown in Table 4, all strains are able to deproteinize shrimp heads with DP more than 60.6% (strain N24). The highest DP was 80.5%, obtained with strain N23. The results show a good deproteinization level in comparison with DP of 73% when using lactic bacteria [22] but less efficace than DP of 83-96% when using proteolytic bacteria [10]. The demineralization levels vary greatly across strains. Strains N11, N21 and N41 exhibited extremely low DA (less than 10%) whereas strain N32 has the highest DA (50.1%), surpassing some proteolytic *Bacillus* bacteria [10] or yeasts [4].

The process of recovering chitin from crustacean by-products is frequently approached by lactic fermentation, which uses bacteria's acid-producing ability to dissolve calcite/carbonate in crustacean shells (demineralization), or by fermentation with bacteria that can produce extracellular proteases, which cleave protein-chitin bonds and release chitin (deproteinization) [23]. In this study, it was found that even the pH of fermented cultures was declined significantly but there is no linear relationship between demineralization levels and pH decrement (results not shown) which show that the demineralization may be not only caused by the acidification during fermentation, but rather related to protein removing process. This co-

Table 3: Sugar catabolism and fermentation ability of isolated yeasts

Strain	Glucose				Saccharose				Lactose			
	Aerobic		Anaerobic		Aerobic		Anaerobic		Aerobic		Anaerobic	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
N11	++	+++	++	-	+++	-	+	+	-	-	-	-
N12	+++	+	++	+	+	-	-	-	-	-	-	-
N13	+++	+	++	-	+++	+++	+	-	+	-	-	-
N21	+++	+	+++	+	+	-	-	-	-	-	-	-
N22	+++	+	+++	+	-	-	+	-	-	-	-	-
N23	+++	+	+++	+	+	-	-	-	-	-	-	-
N24	+++	+	+++	+	+	-	-	-	+	-	-	-
N31	+++	+	++	+	++	-	+	+	-	-	-	-
N32	+++	++	++	+	++	+++	-	-	++	-	-	-
N41	+++	+++	+++	+	+++	-	+	-	-	-	-	-
N42	+++	+	+++	+	-	-	-	-	-	-	-	-

*(-) Not assimilated/ fermented; (+) Weak assimilated/fermented; (++) Moderate assimilated/fermented; (+++) Strong assimilated/ fermented
Source: Authors.

removal was reported for the non-lactic fermentation and was explained by the mineral washing out during protein breaking down [16]. Further studies on extracellular protease production and activities need to be conducted to elucidate this hypothesis. Indeed, the removal of proteins from the protein-chitin network was directly associated with chitin recovery efficiency since the Pearson correlation between these parameters was -0.74 ($p < 0.001$). The strains N24 and N32 showed the highest crude chitin recovery 62.9-64.7% while demonstrated also the lowest deproteinization (60.6-66.1%). The strain N23 had the lowest crude chitin recovery of 38.1% but the highest deproteinization of 80.5%. In all cases, crude chitin recovery was 38.1-64.7%, higher than results reported by using *Bacillus* bacteria for shrimp shell fermentation [22] which was 10.6-40.6%.

CONCLUSION

Eleven yeast strains were isolated and purified from nemchua samples collected in Tho Xuan, Thanh Hoa province. All strains showed good deproteinization capacity, reaching up to 80.5% when cultured on shrimp heads without supplemented nutrients. However, demineralization levels varied widely, ranging from 1.9 to 50.1%. Two strains, N22 and N23, were selected as the most promising candidates as they could deproteinize and demineralize upto about 80% and 40%, respectively, while obtained high chitin recovery yield. Further studies are needed to optimize the removal efficacy, especially on demineralization. Furthermore, the study demonstrates the advantages of the microbial method, which does not require chemicals or thermal energy, making the fermentation process more eco-friendly. These findings highlight Nemchua is a prospective source for yeasts isolation and application in food by-product valorization.

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Table 4: Deproteinization, demineralization and chitin recovery obtained in fermentation with isolated strains

Strain	Deproteinization (%)	Deminiralization (%)	Crude chitin recovery (%)
N11	75.8 ± 1.0^{cde}	6.3 ± 0.2^{a}	50.4 ± 1.7^{ab}
N12	78.7 ± 0.8^{de}	12.1 ± 2.1^{d}	48.4 ± 2.3^{abc}
N13	74.6 ± 0.7^{bcde}	26.5 ± 0.6^{e}	48.5 ± 3^{abc}
N21	71.4 ± 1.9^{bcde}	7.5 ± 0.7^{ad}	51.6 ± 4.4^{ab}
N22	78.5 ± 3.1^{de}	41.4 ± 0.4^{c}	50.5 ± 2.1^{ab}
N23	80.5 ± 3.2^{e}	37.6 ± 0.6^{bc}	38.1 ± 0.0^{a}
N24	60.6 ± 2.7^{a}	37.2 ± 2.2^{bc}	62.9 ± 2.4^{d}
N31	74.4 ± 2.0^{bcde}	29.9 ± 1.2^{ef}	42.1 ± 1.1^{ab}
N32	66.1 ± 2.7^{ab}	50.1 ± 1.9^{g}	64.7 ± 4.9^{d}
N41	67.2 ± 1.7^{abc}	1.9 ± 2.6^{a}	57.0 ± 2.5^{cd}
N42	68.1 ± 2.6^{bc}	34.3 ± 0.7^{bf}	47.7 ± 2.5^{abc}

* The initial pH of medium was 8.87

Source: Authors. Data are presented as Mean ± SD (n=3). Letters (a, b, c, d, e, f, g) indicate statistically significant differences in the same column.

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Bài nghiên cứu

Khảo sát khả năng khử protein và khử khoáng của các chủng nấm men phân lập từ nem chua

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TÓM TẮT
Trong những năm gần đây, việc tận dụng phụ phẩm chế biến thủy sản đã thu hút sự quan tâm đáng kể với mục đích tận dụng các phụ phẩm này như một nguồn biopolymer tự nhiên. Phụ phẩm ngành chế biến tôm, đặc biệt là phần đầu tôm, được công nhận là nguồn dồi dào chitin, một loại polysaccharide tự nhiên có giá trị, được ứng dụng rộng rãi trong nhiều lĩnh vực công nghiệp và y sinh. Nghiên cứu này đã tiến hành phân lập các chủng nấm men bản địa từ Nem chua, một sản phẩm thịt lên men truyền thống của Việt Nam, và đánh giá khả năng thu hồi chitin từ nguồn nguyên liệu đầu tôm thẻ chân trắng (*Litopenaeus vannamei*). Mười một chủng nấm men đã được phân lập và làm thuần thành công từ các mẫu Nem chua, và được đánh giá đặc điểm hình thái và khả năng sử dụng một số carbohydrate cơ bản. Kết quả sàng lọc sơ bộ cho thấy tất cả các chủng phân lập được đều có khả năng tăng trưởng tốt trên cơ chất đầu tôm. Với điều kiện lên men trong 5 ngày với pH ban đầu là 8,87 và mật độ nấm men chủng vào khoảng 10^8 CFU/g, hiệu quả lên men được đánh giá dựa trên các chỉ số: mức độ khử protein, mức độ khử khoáng và hiệu suất thu hồi chitin thô. Kết quả cho thấy tất cả các chủng đều đạt tỷ lệ khử protein trên 60%, trong đó cao nhất đạt 80,5%. Tỷ lệ khử khoáng có sự biến thiên lớn, dao động từ 1,9% đến 50,1%, trong khi pH của dịch nuôi cấy tôm lên men giảm nhẹ từ 8,87 xuống khoảng 8,32–8,80. Hiệu suất thu hồi chitin thô được xác định trong khoảng từ 38,1% đến 64,8%. Mức độ khử protein cho thấy mối tương quan nghịch rõ rệt với hiệu suất thu hồi chitin thô ($r = -0,74$, $p < 0,001$), trong khi không quan sát thấy mối liên hệ rõ ràng nào giữa việc khử khoáng và sự thay đổi pH. Kết quả này cho thấy quá trình loại bỏ khoáng chất có thể liên quan đến việc loại bỏ protein trong quá trình lên men nhiều hơn là chỉ do tác động của sự axit hóa đơn thuần. Trong số các chủng phân lập, N22 và N23 cho thấy tiềm năng ứng dụng công nghiệp lớn nhất, thể hiện qua khả năng khử protein, khử khoáng cao và hiệu suất thu hồi chitin vượt trội. Những phát hiện này nhấn mạnh tiềm năng của việc sử dụng nấm men bản địa trong lên men như một hướng tiếp cận bền vững và "xanh" để nâng cao giá trị phế phụ phẩm chế biến tôm.
Từ khoá: Thu hồi chitin, phân lập nấm men, lên men, đầu tôm, nem chua

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