Recovery of Chitin from Shrimp Waste by Co-fermentation

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Abstract: The large amount of shrimp waste produced every year contains a large amount of natural cellulose material (chitin), which causes a waste of resources. This paper uses co-fermentation to recover chitin from shrimp waste and compares it with other common methods (monomicrobial fermentation, two-step fermentation). By adjusting the inoculation ratio of the Bacillus zanthoxyli strain and Streptococcus thermophilus strain, the ratio of glucose to sucrose, temperature, and pH, we obtained chitin with DP% and DM% of 68.89% and 83.80%, respectively. Through a comparative analysis, we found that DP% and DM% of chitin extracted by co-fermentation were more balanced. The chitin improved by the small-size shrimp shells was DP%, DM% and DA% of 83.76%, 91.48% and 93.47%, respectively, which proved the potential of obtaining the high quality of chitin using the co-fermentation method. Co-fermentation is a viable alternative biological fermentation method for extracting chitin from shrimp waste.

1 INTRODUCTION

The annual global amount of biosynthesized chitin (β(1→4) linked GlcNAc) is approximately 100 billion tons (Ablouh 2020, Zhang 2020). It is the most abundant natural polysaccharide after cellulose and is used as a structural component to support cells and body surfaces (Kumirska 2010, Gbenebor 2017, Balitaan 2020). The world produces approximately 6 to 8 million tons of waste crab, shrimp, and lobster shells annually (Yan, Chen 2015). The global market for chitin and its derivatives include applications in sewage treatment, food and beverages, cosmetics, bioplastics, biomedicine, and agriculture (Casadidio 2019, Abdel-Mohsen 2020, Abdel-Mohsen 2020, Liu 2020). The amount of chitin prepared is approximately 28,000 tons, but its demand exceeds 60,000 tons (Eddya 2020). Therefore, it is urgent to discover a convenient, fast, environmentally friendly, and cost-saving alternative for production.

Shrimp shells are composed of three layers (outer layer, middle layer and inner layer). Chitin is located in the inner layer of the shell and is wrapped with protein. The middle layer is composed of chitin and minerals, and the outer layer contains calcium carbonate and protein (Balitaan 2020, Xin 2020). There are two main methods for extracting chitin: biological extraction and chemical methods. Acids from microorganisms or HCl solutions in chemical methods remove minerals from shrimp shells, whereas proteases or NaOH solutions remove proteins (Marzieh 2019). Deproteinization rate (DP%) and demineralization rate (DM%) are often used as standards of extracted chitin product (Nidheesh, Suresh 2015). The degree of acetylation (DA%) is defined as the average number of GlcNAc units per 100 monomers, expressed as a percentage (Tolaimate 2003). Chitin can deacetylate partially by alkali (50% NaOH) in chemical methods or chitinase in biological extraction (Hamed 2016). When the DA% of chitin is less than 50%, it will dissolve in an aqueous acid solution (pH <6.0), which is chitosan (Kumirska 2010). DA% is the most important factor affecting the application of chitin and chitosan (e.g., biodegradability, chemical modification steps, and solubility). DA% of chitin depends on the raw material and the deproteinization process (Tolaimate 2003). The chitin with high DA% and low protein content is considered as good final products (Marzieh 2019).

Although chemical methods can quickly extract high-quality chitin, such methods reduce the DA%, thereby affecting the crystal structure (Gbenebor 2017), and it is impossible to recover value-added by-

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products, such as proteins and pigments (García 2019). In addition, the molecular weight of the product is low (80–800 kDa), and it cannot be used as an ideal precursor for high-end functional materials (Di 2019). Furthermore, these conventional chemical extraction processes are extremely harmful to the environment, and the high concentration of inorganic acid that is consumed requires a large amount of fresh water for washing after each step (Pachapur 2016, Zhang 2018). Therefore, extracting chitin by biological treatment became a research hotspot. Comparing with chemical methods, chitin obtaining by biological treatment has some advantages, such as better antibacterial activity and biocompatibility, higher molecular weight and crystal index, environment-friendly, low cost (Tanganini 2020). The biological method can recover additional products, such as protein and pigment, and the fermentation broth can be used as feed for aquaculture (Younes 2016, Castro 2018). These values demonstrate that chitin extracted by the biological method has broader application prospects.

So far, the most studied biological method is monomicrobial fermentation. In common bacteria, the DP% and DM% of Bacillus subtilis, Pseudomonas aeruginosa, Lactobacillus plantarum, Bacillus cereus, and Lactobacillus rhamnosus were 84% and 72%, 94% and 84%, 95.3% and 99.6%, 78.6% and 73%, and 30.50% and 83.83%, respectively (Sini 2007, Sorokulova 2009, Sedaghat 2017, Castro 2018, Liu 2020). There are a few strains that can extract high-quality chitin alone (Nidheesh, Suresh 2015), and precious few strains that contain high DP% and high DM% simultaneously. For this reason, some people combine high DM% strains and enzymes or high DP% strains for two-step fermentation. For example, Dun et al. (Dun 2019) combined high-strain Bacillus coagulans and proteinase K to ferment shrimp shells in two steps to obtain high-quality chitin (DP% and DM% are 93% and 91%, respectively); Yongliang Liu et al. [23] used a high DM% (83.83%) strain Lactobacillus rhamnoides and a high DP% strain (83.28%) Bacillus amyloliquefaciens for joint fermentation and obtained relatively ideal products (DP% and DM% are 96.8% and 97.5%, respectively).

Although the fermentation efficiency (DP% and DM%) of the two-step fermentation is higher, the re-sterilization and replacement of the fermentation broth for the second fermentation stage will complicate the operation and cause a waste of resources (Zhang 2021). Therefore, in this study, we tried to explore if the methods of co-fermentation can be used to extracting chitin from shrimp waste, and its performance was compared with the monomicrobial fermentation and two-step fermentation under the same conditions to determine whether this method is advantageous for producing chitin. As far as we know, studies on the obtaining chitin by co-fermentation have not been reported in the literature.

2 MATERIALS AND METHODS

2.1 Materials

Fresh shrimp waste was collected from the Licun Market, and the meat was removed, including head, tail, and legs. The shrimp shells were dried in an oven for 24 h and granulated with a crusher. Then, 2.00 - < 0.20 mm was extracted from it and stored in a reagent bottle at −20℃.

2.2 Bacterial Strains and Culture Conditions

The target strains were isolated from lactic acid fermentation powder and soil obtained from Qingdao University of Science and Technology, and the culture medium was screened with protease strains (lysogeny broth [LB] solid medium supplemented with skimmed milk powder) and an acid-producing strain selection medium (de Man, Rogosa, and Sharpe [MRS] solid medium supplemented with $CaCO_3$) to screen out the strains with larger transparent circles and to select strains, which have the best protease activity, as well as high-yield acid strain, which can be identified by cluster analysis on the sequence of 16S rRNA. The primers used were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTACGACTT-3′).

2.3 Fermentation Conditions and Program Design

Depending on the literature, we chose the initial pH, 5% glucose, 4% inoculum, 3% shrimp shell, and 120 rpm as the initial fermentation conditions (Zhang 2012, Sedaghat 2017, Liu 2020) and reset the vaccination plan on this basis: (1) the strains producing high quality chitin in co-fermentation method were used to explore the two-step fermentation method and monomicrobial fermentation method (fermentation time was 6 days); (2) the fermentation conditions were optimized for different fermentation methods (fermentation time

was 3 days); by comparing and analyzing the DP% and DM% after fermentation, the best biological fermentation plan was determined (fermentation time was 6 days); (4) the small-sized shrimp shells were fermented by the optimal fermentation method (fermentation time was 6 days), and the products were characterized by FT-IR.

2.4 The Analysis of Ash Content

Fresh shrimp shells were dried in a drying oven at 105℃ for 48 h, and the moisture content was calculated by the weight difference before and after drying (Bellaaj 2012). The ash content was measured by heating in a muffle furnace at 550℃ for 4 h (Sedaghat 2017).

2.5 The Content Measurement of Total Protein

The total protein, which in shrimp shells was calculated by subtracting the nitrogen content in chitin by the total nitrogen content, was measured using a Kjeldahl Nitrogen Analyzer (JK9830; Jinan Jingrui Analytical Instruments Ltd., China) (Liu 2020).

2.6 The Determination of Protease Activity

The protease activity was determined according to the method described by *YaohaoDun et al*. (Dun 2019). In short, casein was used as a substrate, and fermentation broth and phosphate buffer (pH = 7.5) were added to induce a reaction at 30℃ for 10 min. The reaction was terminated immediately with trichloroacetic acid, and a color reaction with Folin reagent was induced at 40℃ for 20 min. The absorbance was measured at 680 nm. The protease activity unit, which was expressed in U/ml, was defined as the amount of 1 μg tyrosine produced by 1 ml liquid enzyme hydrolyzing casein in 1 min.

2.7 The Determination of DP% or DM%

The DP% or DM% was calculated by the equation:

$$Y\% = ((C_1 \times W_1) - (C_2 \times W_2))/(C_1 \times W_1)$$

Y% is DP% or DM%; C_1 is the protein or ash content before fermentation; W_1 is the protein or ash dry weight before fermentation; C_2 is the protein or ash content after fermentation; and W_2 is the protein or ash dry weight after fermentation.

2.8 Characterization of Chitin

A Fourier transform infrared spectrometer (FT-IR) was used to study chitin, and the DA% of chitin was calculated based on the FT-IR (Knidri 2016).

The FT-IR was calculated by the equation:

$$DA\% = (A_{1655}/A_{3450}) \times 100/1.33$$

2.9 Statistical Analysis

All experimental data are observed in triplicate, and the means ± standard deviations were reported. Statistical analysis was conducted using SPSS version 17 software. Statistical significance was determined at $P < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Strain Screening

Nine protease-producing strains and three acid-producing strains with relatively large transparent circles were selected from the screening medium of the protease strain and the acid strain.

Protease activity and pH were measured 24 h after fermentation in LB and MRS broth.

Three strains (2, 4, and 7) with higher protease activity were selected and named B1, B2, and B3, respectively (Table 1). Homologous strains were searched in the NCBI library using 16S rRNA. The homologies of strains B1, B2, and B3 with *Bacillus mobilis*, *Bacillus zanthoxyli*, and *Bacillus proteolytic strains* is as high as 99% or more. The three acid-producing strains that were identified as the same strain and have more than 99% homology with *Streptococcus thermophilus* strain were named L.

Table 1: Protease activity of enzyme-producing strains and pH of acid-producing strains.

numbers	Protease activity (U/ml)	pH
1	1.01 ± 0.22	
2	6.55 ± 0.58	
3	1.01 ± 0.63	
4	3.22 ± 0.58	
5	2.54 ± 0.15	
6	2.77 ± 0.1	
7	4.15 ± 0.63	
8	1.46 ± 0.29	
9	2.19 ± 0.19	
10		4.97 ± 0.02
11		4.72 ± 0.02
12		4.9 ± 0.01

3.2 Screening of Fermentation Strains

We found that the fermentation efficiency of all screened strains was relatively lower than previously reported (Sini 2007, Castro 2018). In our study, the highest DP% and DM% of the B2 strain were only 39.95% and 58.46%, respectively (Figure 1. A). It may be that the size of our shrimp shells will not allow acids and proteases to completely contact the reactants (Abdelmalek 2017). To verify this conjecture, we used the B2 strain to ferment shrimp shells of different sizes. The result shows that the fermentation efficiency in shrimp shells gradually increased with the decreasing shrimp shell size and reached the maximum at 0.45–0.3 mm (Figure 1. E). The reason for the lower fermentation efficiency of less than 0.3 mm is that the smaller size of the shrimp shells facilitates aggregation in the triangular flask during the fermentation process. In addition, the smaller the shrimp shell size, the greater the loss during washing. Therefore, shrimp shells with a particle size of 2.00-1.43mm were used in the fermentation exploration stage.

In the process of co-fermentation (Figure 1. B, C), the DM% of the group with L bacteria was higher than that of other groups ($P < 0.05$), but the DP% was not significantly different ($P < 0.05$). The DP% and DM% of B2-L were the best, at 17.04% ($P < 0.05$) and 79.89% ($P < 0.05$), respectively. In the three-strain and four-strain group, B1-B2-B3-L showed the highest DM%, which was 81.82%, but the fermentation conditions were more regulated than those of B2-L. The other groups (excluding L) had lower fermentation removal efficiency than had B2, which did not show the potential to justify further research. After comprehensive consideration, we chose B2 and L as the fermentation strains for co-fermentation.

The performance of B2 and L strains in two-step fermentation were further explored. Figure 1.D shows that fermentation efficiency of the replacement broth group (C) was higher than that of the non-replacement broth group (NC), and the results of B2→L were more balanced than those of L→B2. We found that the fermentation efficiency of during two-step fermentation tends to depend on the strain used in the first fermentation stage, which deviated from the results of *Liu, Y et al.* (Liu 2020). The reason for this phenomenon may be that the fermentation broth of the second fermentation stage cannot provide sufficient growth for bacteria or that the exposed chitin in the shrimp shells inhibits the growth of bacteria. In summary, we chose the B2→L-C group for the two-step fermentation method for comparison with the B2-L fermentation method.

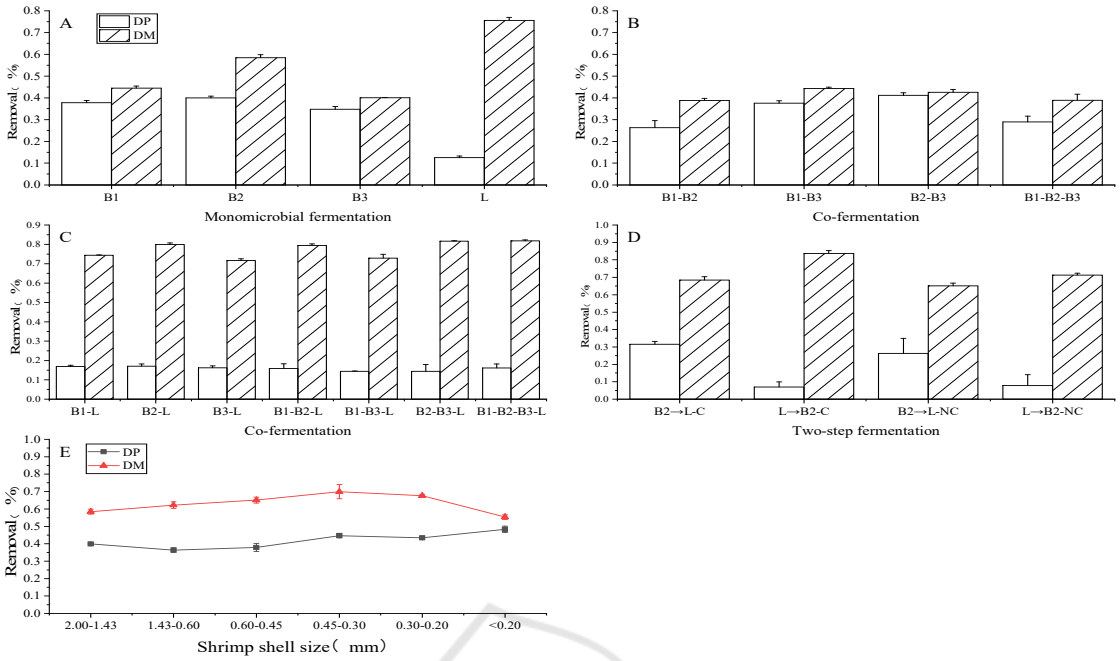


Figure 1: Selection of fermentation strains.

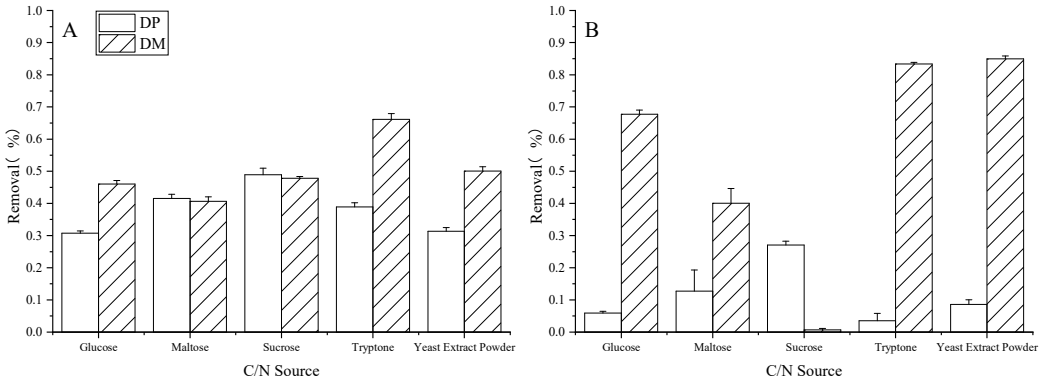
A, B, C, D, E: Residual protein and mineral content of chitin after fermentation; D: C means replacing the fermentation broth, NC means using the fermentation broth of the previous strain for fermentation

We chose B2 and L strains as the research objects of co-fermentation, which can be compared with the fermentation results of B2, L, and B2→L-C. Under the initial conditions, we determined that the fermentation of the B2 strain was the most balanced, and the DM% of B2-L was the best.

3.3 The Optimization of Monomicrobial Fermentation and Two-step Fermentation

There are two indicators that the extracted chitin needs to satisfy during the final evaluation process: residual protein content and residual mineral content. In the regulation of fermentation conditions, some values have only the highest DM% or DP%. As shows in Figure 3. D, DP% was the highest at 2.5% sucrose content, but its DM% was the lowest, which leaded to conflicts when we chose a certain fermentation condition. We could not guarantee that DM% and DP% were both at the optimal highest proportion. Therefore, when selecting DP% and DM%, it was not only required to have a relatively small difference, but also a relatively high value, as shows in Figure 5. B.

To better explored the fermentation of co-fermentation, we first needed to explore the fermentation conditions of single bacteria. Figure 2. shows that carbon (C)/nitrogen (N) sources have a greater impact on monomicrobial fermentation. Among them, after sucrose was added, the DP% of the B2 and L strains increased significantly ($P < 0.05$), but the decrease of the DM% of the L strain from 67.73% to 0.63% ($P < 0.05$) was not suitable for use as a C source. Tryptone and yeast extract powder were suitable for B2 and L strains, respectively. In summary, the B2 strain uses sucrose and tryptone as C/N sources, whereas the L strain uses glucose and yeast extract powder.



A: B2 strain; B: L strain

Figure 2: Optimization of C/N sources for B2 and L.

After the C/N source was determined, the B2 strain was optimized for temperature, inoculum, pH, and sucrose content. The results show (Figure 3. A, D) that the temperature and sucrose content have a greater impact on the B2 strain. We chose relatively high and balanced conditions of DP% and DM% for the fermentation conditions of B2, and the fermentation conditions of high DP% were used for the conditions of the B2 strain in two-step fermentation. Therefore, the optimized result of strain B2 was 7.5% sucrose, 2% tryptone, 30℃, 2% inoculum, and pH 7.5, and the optimized result of the first fermentation as part of a two-step fermentation process was 2.5% sucrose, 30℃, 2% inoculum, and pH 7.5.

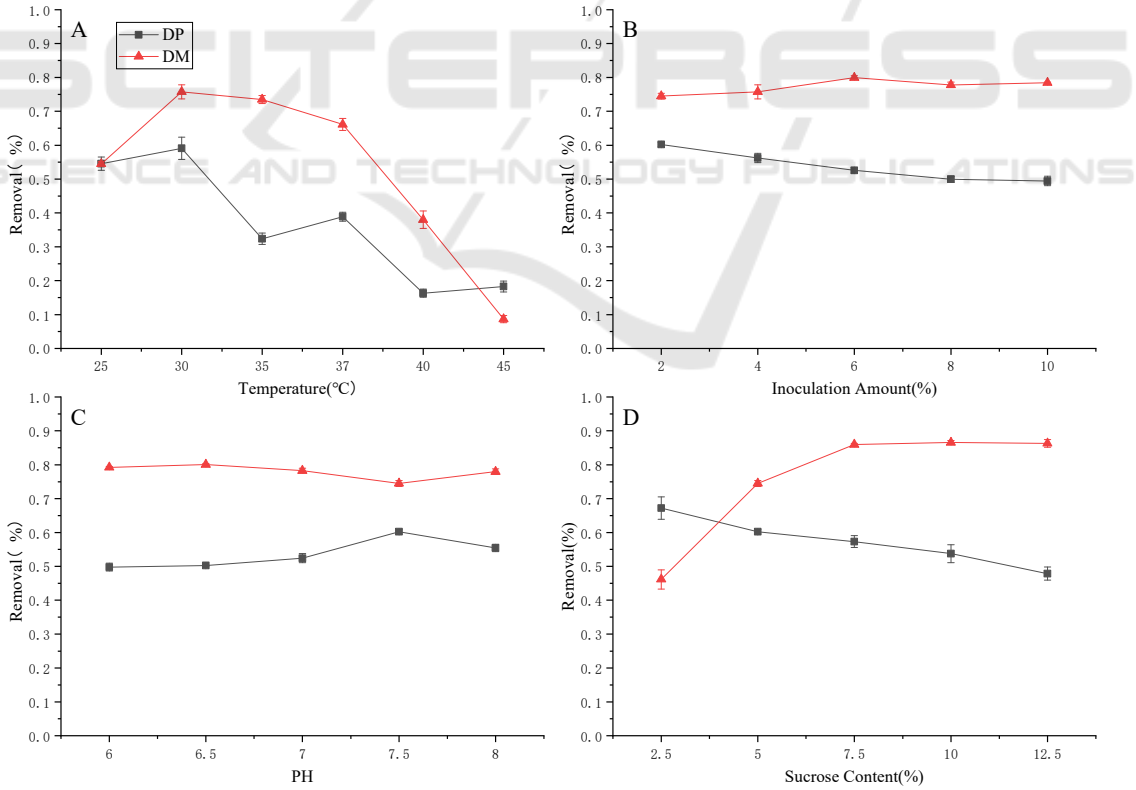


Figure 3: Optimization of fermentation conditions of B2 strain.

Only the temperature of the L strain had a greater influence on DM% (Figure 4. A). The selection of fermentation conditions was the same as that for the B2 strain. Therefore, the optimized result of the L strain was 5% glucose, 2% yeast extract, 30°C, 4% inoculum, and pH 6.0; as the second fermentation strain in the two-step fermentation process, the fermentation conditions were 10% glucose, 2% yeast extract powder, 30℃, 4% inoculum, and pH 6.0.

According to previous reports in literature (Zhang 2021), the two strains in the two-step fermentation play different roles, one of which is responsible for DP and the other is responsible for DM. In this experiment, B2 with high DP% fermentation conditions was mainly responsible for the deproteinization of shrimp shells, and the L strain with high DM% fermentation conditions was used to demineralization. The final fermentation conditions of B2→L-C were as follows: The first stage (B2 strain: 2.5% sucrose, 30℃, 2% inoculum, pH 7.5), the second stage (L strain: 10% glucose, 30℃, 4% inoculum, pH 6.0, 2% yeast extract powder).

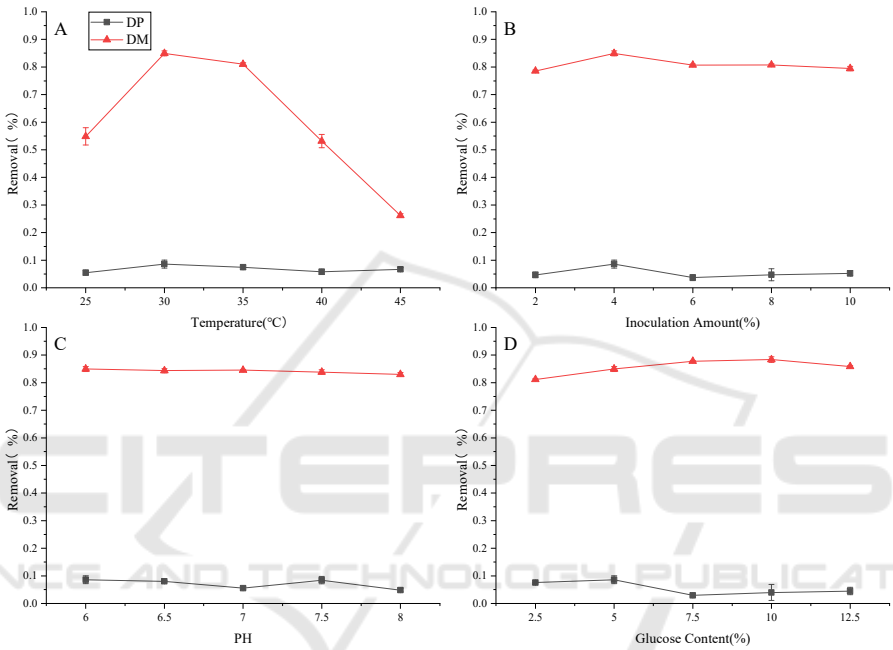


Figure 4: Optimization of fermentation conditions of L strain.

3.4 The Optimization of the Co-fermentation Conditions

Co-fermentation explores the ratio of each strain and then considers how different strains may be adapted to C sources. The DP% of B2-L (1:1) was too low, so we attempted to increase the DP% of co-fermentation by adjusting the ratio of B2 strains. Interestingly, as the proportion of B2 strains increases, DP% gradually increases and then stabilizes after 1:4. At 1:6, the fermentation efficiency of chitin was optimal and most balanced, although it was the same as that of B2 ($P < 0.05$). We think there is no value in continuing to explore, so we continued to use 1:1. We mentioned in Section 3.3 that the B2 strain prefers sucrose, whereas the L strain prefers glucose. On this basis, we adjusted the ratio of sucrose and glucose. The results show (Figure 5. B) that when the ratio of glucose to sucrose was 1:1, the fermentation efficiency (DP% = 52.49%, DM% = 43.93%) was better than the fermentation effect of the B2 strain.

Since the optimal temperature of strain B2 and strain L was the same, and the additional N source would reduce the DP% (figure 3 and figure 4), we only discussed changes in the inoculum, pH, and C source content. Interestingly, the amount of inoculation also had a huge impact on the results. After the inoculation amount reached 6%, the fermentation efficiency was similar to the unoptimized result ($P < 0.05$). It is very likely that the increase of the L strain inhibited enzyme production of the B2 strain or inhibited the deproteinization of protease. The final fermentation conditions of B2-L were B2:L inoculated at a ratio of 1:1, a ratio of glucose to sucrose of 1:1, 4% inoculation, and pH 7, temperature 30℃.

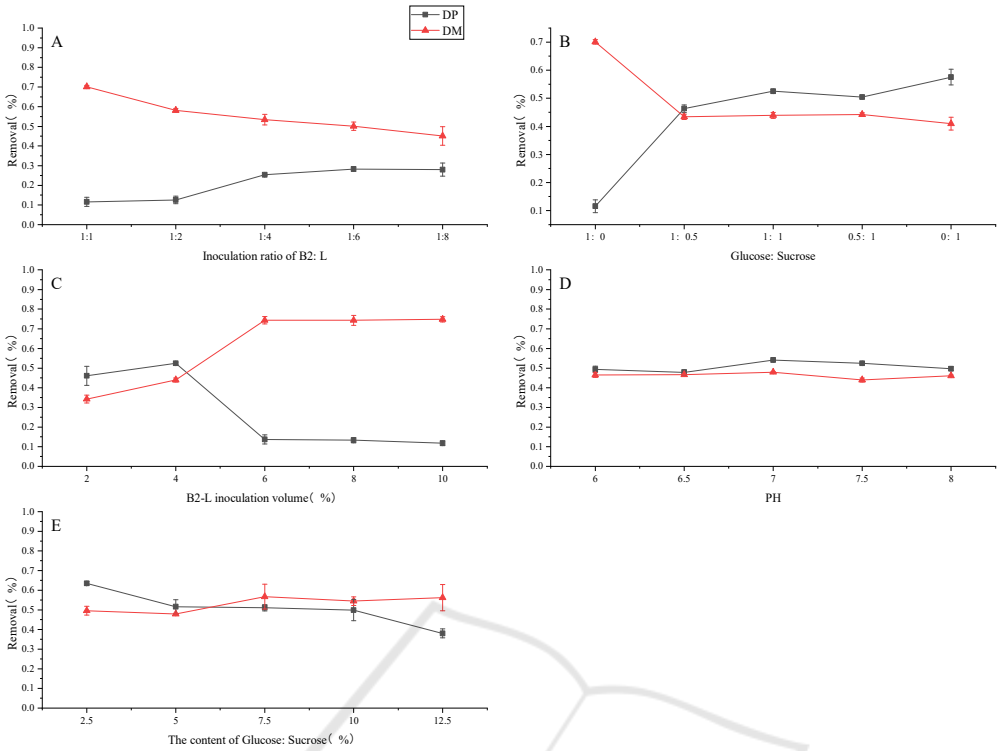


Figure 5: The optimized fermentation conditions of B2-L and the optimized results of three fermentation methods.

3.5 Comparison of Three Fermentation Methods

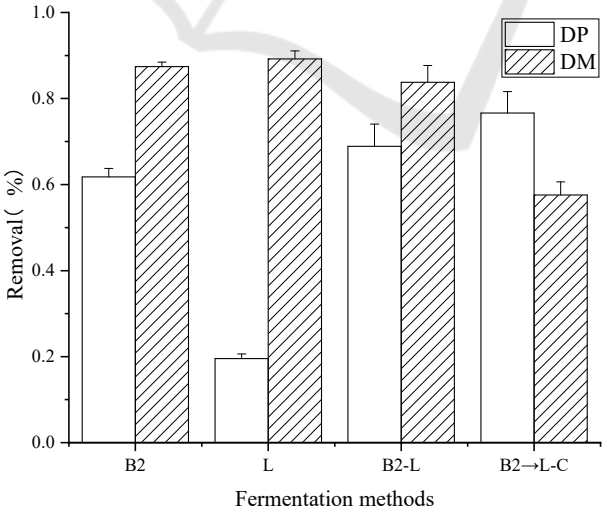


Figure 6: The optimized results of the three fermentation methods.

Although the optimized L had the highest DM% (89.19%, P<0.05, figure 6.), its DP% was only 19.55%. There are too many protein residues in chitin, so the L strain was not suitable for extracting chitin from shrimp shells by fermentation alone.

The DP% (76.64%) of the B2→L-C group was the highest (figure 6.), which was different from the initial fermentation results (figure 1. D). The reasons for this phenomenon were that we purposely increased the deproteinization efficiency of B2 strains by optimization of fermentation conditions. Although additional nutrients (C/N sources) could be added to make up for the second stage fermentation of the strain, this would also increased the cost of chitin extraction. In addition, wastewater discharge and energy consumption would also occur when the fermentation broth was replaced (Zhang 2021). Therefore, the B2 and L strains in this experiment were not suitable for the production of chitin from shrimp shells using the two-step fermentation method.

The DP% and DM% of the optimized B2 strain were 61.78% and 87.41% (figure 6.), respectively, and there was a big difference between the DP% and DM% (25.63%). Nevertheless the difference between the DP% and DM% of the B2-L group (the DP% and DM% is 68.89% and 83.80%) was the smallest (14.91%), so its quality of fermentation product was the highest when comparing the monomicrobial fermentation and the two-step fermentation.

Although the regulation process of co-fermentation (B2-L) is complicated, there are more fermentation conditions that can be regulated than monomicrobial fermentation. For example, co-fermentation can optimize the ratio of different strains and the ratio of different carbon sources. Therefore, there are more opportunities to find suitable fermentation conditions, and its potential to produce high-quality chitin is higher. Compared with the two-step fermentation method, the co-fermentation operation was simple, and it only needed to complete the whole fermentation in one step (Zhang 2012). Therefore, the co-fermentation method for preparing chitin is a potential fermentation method.

3.6 The Optimal Fermentation Results for B2-L

Co-fermentation (B2-L), compared with the other two fermentation methods, was considered to be the best fermentation method. In order to further improve the quality of its product, the effect of shrimp shell size was studied (figure 7). The fermentation bottle was vigorously shaken once a day in view of the tendency of small-size shrimp shells to aggregate. The results shows that the DP% and DM% could reach to 83.76% and 91.48%, respectively when reduced the size of shrimp shell (<0.2mm, figure 7), which indicated the great potential of co-fermentation.

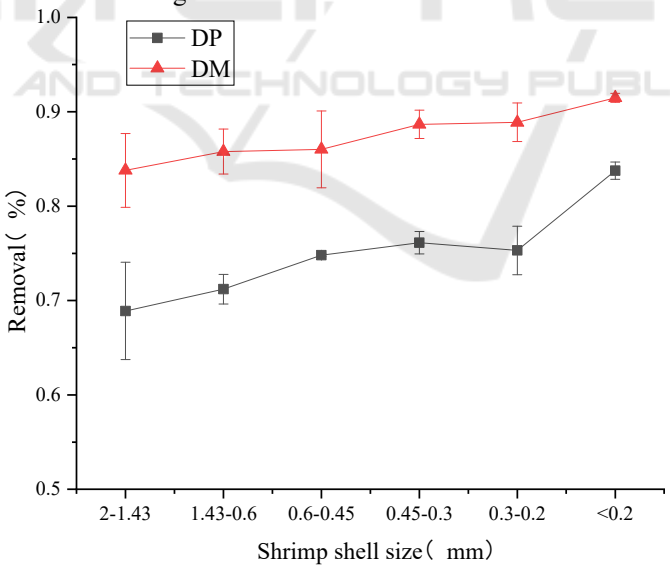


Figure 7: The fermentation efficiency of different sizes of shrimp shells in B2-L.

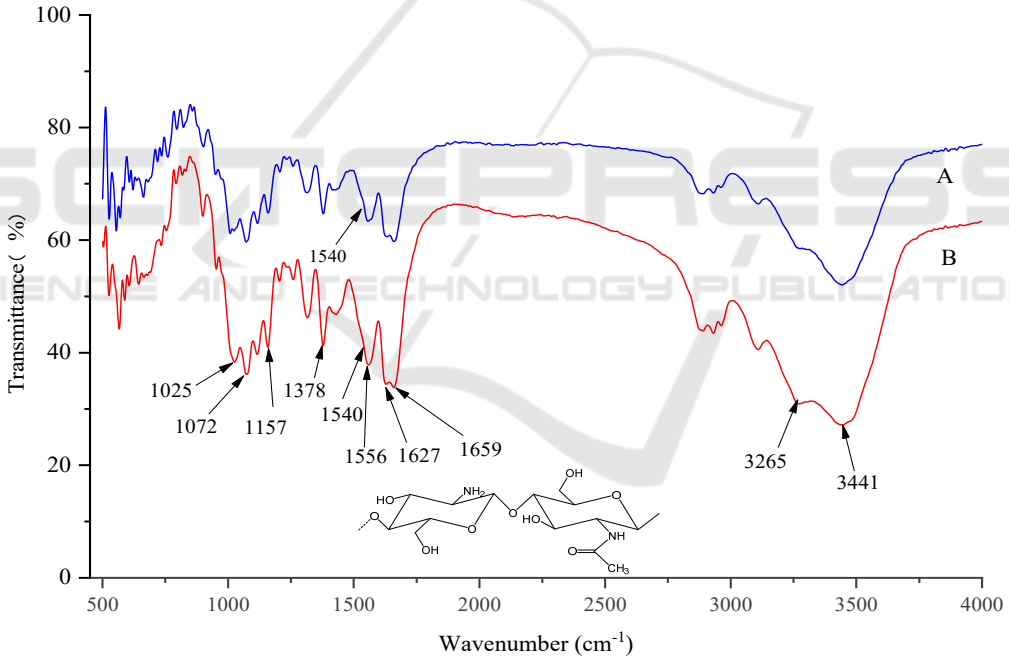
Sini T K et al. (Sini 2007) showed that the DP% and DM% of chitin obtained from the fermentation of shrimp shells by *Bacillus subtilis* were 84% and 72%. Compared with it, the DM% increased by 19.48% after co-fermentation of B2 and L (DP% = 83.76%, DM% = 91.48%). The latest report showed that the two-step fermentation of *Bacillus subtilis* and *Acetobacter pasteurianus* can produce chitin with DP% and DM% of 94.5% and 92.0% (Zhang 2021). The DP% in this trial was 10.5% lower than the

former report. The DP% and DM% of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus mojavensis* reported by *Hajji, S et al.* (Hajji 2015) were 81.6% and 76.6%, 83.1% and 81.8%, 80.4% and 73.2% respectively, which were lower than the DP% and DM% of chitin in this experiment. This showed that the co-fermentation is a potential and effective method for preparing chitin. The reason why their fermentation result is lower than the former report may be the weak DP ability of L strain. Therefore, when selecting dominant strains, we must pay attention to selecting strains with better acid-producing ability and protease-producing ability.

3.7 Fourier Transform Infrared (FT-IR) Spectra Analysis and Determination of the Degree of Acetylation

Figure 8. showed that the FT-IR of chitin prepared from shrimp shells (<0.2mm) by co-fermentation of B2 and L was similar to that of commercially available chitin. The prepared chitin had the typical characteristic peaks of α-chitin, including amide I bands about 1659 cm^{-1} and 1627 cm^{-1} (C=O stretching vibration), amide II bands at 1556 cm^{-1} (N-H bending vibration), and amide III at bands 1378 cm^{-1} (C-N stretching vibration). The stretching vibrations of -OH and -NH appeared at 3441 cm^{-1} and 3265 cm^{-1}. The other peaks of the chitin structure were 1157 cm^{-1} (C-O-C asymmetric vibration), 1025 cm^{-1} and 1072 cm^{-1} (C-O stretching vibration), and 1378 cm^{-1} (C-H shear vibration). This was the same as described by El *Knidri, H. et al* (Knidri 2016). The spectrum of chitin prepared by the co-fermentation method and commercial chitin was lacking the absorbance peak at 1540 cm^{-1}, where proteins would normally give rise to absorption (Liu 2020).

The DA% of chitin prepared by B2-Lwere 93.47%, which is higher than that of chitin prepared by commercial (86.37%) and *Manni, L et al.* (89.50%) (Manni 2010).



A: Commercial chitin.
B: Chitin prepared by mixed-bacteria simultaneous fermentation method (B2-L).

Figure 8: FT-IR spectra of chitin.

4 CONCLUSIONS

The DP%, DM% and DA% with 83.76%, 91.48% and 93.47% of chitin were co-fermentation of B2 and L, respectively. Comparing to the monomicrbial fermentation and the two-step fermentation, the co-fermentation can be used to extract chitin from shrimp waste, which provides a feasible fermentation method for the large-scale production of chitin in the future.

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