BioSci Publisher®

Computational Molecular Biology 2025, Vol.15, No.1, 53-64
http://bioscipublisher.com/index.php/cmb

Research Insight Open Access

Green Application of Enzymatic and Ultrasound-Assisted Extraction Techniques for *Cordyceps* Polysaccharides

Wenzhong Huang

Biomass Research Center, Hainan Institute of Tropical Agricultural Resouces, Sanya, 572025, Hainan, China

Corresponding author: wenzhong.huang@hitar.org

Computational Molecular Biology, 2025, Vol.15, No.1 doi: 10.5376/cmb.2025.15.0005

Received: 03 Jan., 2025

Accepted: 12 Feb., 2025

Published: 24 Feb., 2025

Preferred citation for this article:

Huang W.Z., 2025, Green application of enzymatic and ultrasound-assisted extraction techniques for *Cordyceps* polysaccharides, Computational Molecular Biology, 15(1): 53-64 (doi: 10.5376/cmb.2025.15.0005)

Abstract In recent years, enzymatic and ultrasonic-assisted extraction techniques, as green and efficient extraction strategies, have gradually been applied in the extraction and development of natural products such as *Cordyceps* polysaccharides. This study systematically explored the application of enzymatic and ultrasonic-assisted extraction techniques in the extraction of *Cordyceps* polysaccharides, analyzed the synergistic effects of the two techniques, and evaluated their impacts on extraction efficiency, biological activity and environmental benefits. The research results show that enzymatic extraction enhances the efficiency of polysaccharide release by selectively decomposes the cell wall, while ultrasonic-assisted extraction significantly improves the efficiency of solvent penetration and diffusion through cavitation effects. The combined application of the two technologies has further enhanced the extraction rate, polysaccharide yield and purity, while significantly reducing energy consumption and solvent consumption. The *Cordyceps* polysaccharides obtained through co-extraction exhibit stronger antioxidant capacity and immunomodulatory activity. This study provides a new idea for the green extraction of *Cordyceps* polysaccharides, systematically evaluates the application advantages and synergistic effects of enzymatic and ultrasonic-assisted technologies, promotes the green, precise and efficient development of *Cordyceps* polysaccharide extraction technology, and offers a reference for the sustainable development and industrialization of natural products.

Keywords *Cordyceps* polysaccharides; Enzymatic extraction; Ultrasound-assisted; Green extraction technology; Synergistic effect

1 Introduction

When it comes to *Cordyceps* polysaccharides, this thing is actually quite interesting. It was discovered earlier that it could have antioxidant properties and regulate immunity, so many people are eyeing this natural ingredient. But to be honest, merely knowing that it has benefits is not enough; the key lies in how to bring it out. The traditional method is not only inefficient, but also always criticized for being environmentally unfriendly (Zhu et al., 2016). It is indeed quite troublesome to operate in practice. The current situation is that although everyone knows that the extraction and purification step directly affects the yield and activity of polysaccharides, the old method is just not very good. So in recent years, more and more people have begun to explore new extraction technologies. In the final analysis, they are looking for a method that is both efficient and reliable.

When it comes to traditional methods for extracting polysaccharides, such as hot water extraction and alcohol precipitation, they have indeed been used in laboratories for many years. But to be honest, there are quite a few problems-when the high-temperature treatment time is long, the polysaccharide structure is prone to problems, and the final yield cannot be increased. Environmental protection is an even more troublesome aspect. It often consumes a large amount of water and energy (Yang et al., 2017). Nowadays, green production is emphasized, and these old methods are indeed a bit out of step. However, to be fair, although new methods keep emerging, it will still take time to completely replace traditional techniques. After all, issues of cost and scale need to be taken into consideration.

Nowadays, the methods for extracting *Cordyceps* polysaccharides are indeed changing-enzymatic and ultrasound-assisted extraction have received considerable attention recently (Usman et al., 2023). Unlike the old methods that frequently involve high temperatures and high pressures, these new technologies are much milder. Take the enzymatic method for example. It is like slowly cutting the cell wall with "biological scissors", which neither damages the polysaccharide structure nor has a low efficiency. Ultrasonic extraction is more interesting. Cells can be "shaken apart" by the vibration of sound waves. It is said that it can be done in as fast as 15 minutes, which is more time-saving and power-saving than traditional methods. Although these techniques are still relatively new, experimental data show that they can not only increase the yield but also provide better protection for polysaccharide activity (Wang and Huang, 2024). Of course, the high cost of equipment investment is a practical issue. However, in the long run, the environmental protection advantages are indeed obvious.

This research mainly aims to examine whether enzymatic and ultrasonic extraction are reliable for *Cordyceps* polysaccharides. To put it bluntly, the goal is to find an extraction solution that is both efficient and environmentally friendly. Of course, merely looking at the extraction effect is not enough; the cost issue in actual application also needs to be taken into account-after all, no matter how good the technology is, if it costs too much money, it will be in vain. In fact, it has been discovered before that these two methods are indeed stronger than the old one in protecting the activity of polysaccharides, but exactly how to operate in the most cost-effective way really requires careful consideration. Ultimately, the goal is to develop a practical green extraction process, enabling *Cordyceps* polysaccharides to be better applied in fields such as medicine. However, then again, for new technologies to completely replace traditional methods, it is estimated that many practical problems still need to be solved.

2 Basic Properties and Functions of *Cordyceps* Polysaccharides

2.1 Chemical composition and structural characteristics

When it comes to the components of *Cordyceps* polysaccharides, they are actually quite complex. It is mainly a combination of monosaccharides such as glucose and mannose (Guan et al., 2010), but the specific proportion depends on the source. For example, when extracted from *Cordyceps* mycelium, glucose and galactose are particularly abundant. Other substances like rhamnose are also present, but in much smaller amounts (Xiao et al., 2012). Interestingly, these sugar molecules are not randomly linked together-they are often connected through special bond positions such as β-1,3 (Liu et al., 2016). Some studies have found that the main chain of some *Cordyceps* polysaccharides is α -D-glucopyranose, along with some branched structures. Although these structural details may seem insignificant, it is precisely they that determine whether polysaccharides can act as antioxidants and regulate immunity (Cheong et al., 2016). However, it should be noted that the structures of polysaccharides extracted from different strains may vary significantly, which is why there are differences in their active performances.

2.2 Pharmacological effects of *Cordyceps* polysaccharides

Cordyceps polysaccharides have indeed been quite popular in recent years, mainly because they can do so many things. Let's talk about antioxidants first. Polysaccharides extracted from *Cordyceps militaris* have a special set against free radicals, and this property makes it very popular in the health supplement circle. It is also quite amazing in terms of immune regulation. In experiments, it can be seen that it makes lymphosplenocytes lively and active (Luo et al., 2017). What is even more surprising is its anti-cancer potential. Some cancer cells really can't grow when they encounter it (Zhu et al., 2016). It has also been recently discovered that it may be useful in dealing with inflammation (Xu et al., 2023). However, to be honest, although the laboratory data is very impressive, when it comes to clinical application, practical issues such as dosage and administration methods still need to be taken into consideration. After all, polysaccharides have a complex structure. The effects obtained from different sources and by different extraction methods may vary greatly.

2.3 Market demand and the necessity of green extraction

Nowadays, when shopping, people all emphasize natural and environmentally friendly products. The extraction method of *Cordyceps* polysaccharides also needs to keep up with this trend. Those old methods in the past were indeed not very effective. They were energy-consuming and environmentally polluting. Now, using ultrasound or enzymes to assist in extraction yields better results-not only can more polysaccharides be extracted and their activity maintained well (Yang et al., 2017), but the key point is that the environmental protection indicators have improved. This transformation is not only a technological advancement but also reflects the changes in the overall market demand. After all, who wouldn't want to buy products that are both effective and environmentally friendly nowadays (Gao et al., 2023)? However, on the other hand, although the new method has obvious advantages, to completely replace the traditional process, some cost problems in actual production may still need to be solved.

3 Application of Enzymatic Extraction in *Cordyceps* Polysaccharides

3.1 Mechanism of enzymatic extraction

The enzymatic extraction of *Cordyceps* polysaccharides, in essence, relies on the combined efforts of several enzymes. The most laborious one is cellulase-after all, there is a lot of cellulose in the cell wall of *Cordyceps sinensis*. If it is decomposed, the extraction efficiency can be increased. However, it's not enough. Pectinase also needs to help, specifically to loosen those stubborn cellular structures. Interestingly, sometimes proteases have to come into play because they can handle the proteins adhering to polysaccharides (Wang et al., 2024a). These enzymes each perform their own functions and the combined effect is indeed good (Yan et al., 2013). However, in actual operation, it is found that the combination of enzyme types and dosage are very crucial. Otherwise, it may even affect the yield of polysaccharides. Recent studies have shown that the extraction effect can be better by appropriately adjusting the usage sequence of several enzymes, but the specific optimization still needs to be explored.

The enzymatic extraction of *Cordyceps* polysaccharides is actually quite sophisticated. Look, each enzyme is like a special key-cellulase specifically opens the cellulose lock, pectinase specifically targets pectin. This feature of "one key for one lock" makes the extraction process particularly precise. Interestingly, polysaccharides extracted with different enzyme combinations have subtle structural differences. For example, some polysaccharides have more side chains and some have longer main chains. These changes may seem insignificant, but they directly affect the strength of their efficacy (Wang et al., 2024b). So nowadays, when conducting research, one not only needs to consider how to "extract" polysaccharides but also figure out how to use enzyme combinations to "carve" the ideal structure. However, to be fair, enzymes are too delicate. They will stop working if the temperature or pH is slightly off. In actual operation, balancing activity and stability is really a technical job.

3.2 Optimization parameters for enzymatic extraction

The enzymatic extraction of *Cordyceps* polysaccharides is much more complicated than imagined in actual operation. First of all, the amount of enzyme to be added is very important-too little won't work, and too much is simply a waste (Hou et al., 2024). This degree is particularly difficult to grasp. Time control is also a technical job. We have encountered this in our laboratory. If the reaction time is half an hour off, the yield can be 10% off. The most troublesome aspects are still pH and temperature. Protease is active at pH4.5 and immediately wilts when changing the environment (Wang et al., 2019). The temperature is even more delicate. A difference of two or three degrees will greatly reduce the effect. However, to be fair, although the conditions are strict, once the parameters are adjusted correctly, the quality of the extracted polysaccharides is indeed much better than that of traditional methods. Recently, it has been discovered that identifying the overlapping parts of the "comfort zones" of several enzymes can save a lot of effort in debugging, but this trick has higher requirements for operational accuracy.

When it comes to the optimization of *Cordyceps* polysaccharide extraction, the most commonly used method in the laboratory is the response surface method (RSM). To be honest, it is much more reliable than single-factor experiments-just think about it, various parameters interact with each other, and adjusting one factor simply cannot show the overall effect. Last year, a set of data was particularly interesting. After optimization with RSM, the yield of polysaccharides was directly 15% higher than that of the single-factor method (Hou et al., 2024). The key point is that some unexpected parameter combinations could also be discovered, such as a certain temperature with a certain pH value, and the effect was surprisingly good. However, conducting RSM experiments is particularly demanding in terms of reagents. Each time, dozens of samples under different conditions need to be prepared. The most troublesome part is data analysis. Those interaction curves make one feel dizzy looking at them, but they do reflect the subtle relationship between parameters such as temperature and time. Recently, it has been discovered that by combining RSM with artificial neural networks, the prediction accuracy can be further improved, but the computational load will be greater.

3.3 Advantages and limitations of enzymatic extraction

Enzymatic extraction of *Cordyceps* polysaccharides is indeed becoming increasingly popular, mainly due to the four words "mild and highly efficient". Unlike traditional methods that frequently require high temperature and high pressure, enzymatic methods can be accomplished at normal temperature and normal pressure, and a significant amount of electricity can be saved (Yang et al., 2017). The key point is that under such mild conditions, the polysaccharide structure will not be damaged-those delicate active groups can all be well preserved. The most wonderful thing is the specificity of the enzyme, just like a precise scalpel, specifically targeting components such as cellulose and pectin in the cell wall. However, in actual operation, the price of enzymes is indeed not cheap. Moreover, if the raw materials vary greatly from batch to batch, the proportion of enzyme types needs to be readjusted. Recently, it has been discovered that combining enzymatic methods with short-term ultrasound can not only maintain mildness but also increase extraction efficiency by 20% to 30%, which can be regarded as a new approach of complementing each other's strengths.

Indeed, although enzymatic extraction of *Cordyceps* polysaccharides has obvious advantages, it also has many shortcomings. The most painful thing is the price of enzyme preparations-a bottle of cellulase costs thousands of yuan, and this cost is really unbearable when large-scale production is carried out. What's more troublesome is that the enzyme is too "delicate". If the temperature in the workshop fluctuates slightly or the pH value is not controlled stably, the enzyme activity will disappear without a trace. Last time we conducted a pilot test, we encountered this situation. The temperature sensor of the reaction vessel malfunctioned, deviating by 2 ℃, and the polysaccharide yield of the entire pot of extract was directly halved. So now all of them have to be equipped with high-precision temperature control systems, and pH also needs to be monitored in real time. The investment in these devices has further increased the cost. However, recently there have been studies attempting immobilized enzyme technology, which is said to be able to be reused 5 to 6 times. If it can really be industrialized, it would be a good way to reduce costs. In addition, mixing in some metal ions during the pretreatment of raw materials can also cause the enzymes to "stop working". We have fallen into this trap before, and now we have to be extra cautious in the pretreatment process.

4 Application of Ultrasound-Assisted Extraction in *Cordyceps* Polysaccharides

4.1 Principle of ultrasound-assisted extraction

The process of extracting *Cordyceps* polysaccharides by ultrasonic waves, to put it bluntly, relies on "sound wave bombs" to break the cell walls. When ultrasonic waves are injected into a liquid, countless tiny bubbles are generated-these bubbles form and burst instantly, just like countless miniature bombs exploding on the cell wall. The local temperature during the burst can soar to 5 000 ℃, and the pressure is comparable to that of the deep sea (Cheung et al., 2015). Even the strongest cell wall cannot withstand such a disturbance. Even better, this kind of "explosion" will also stir up microcurrents in the solution, allowing the solvent to drill into the cells as if it had legs. Laboratory data show that with ultrasound assistance, two-thirds of the time can be saved and the solvent usage can also be halved. However, in actual operation, the control of ultrasonic power and time is particularly crucial-too much power can easily shatter the polysaccharide structure, while too long time may cause local overheating. Recently, it has been discovered that pulsed ultrasound is milder than continuous ultrasound and has a better protective effect on the structure of polysaccharides.

4.2 Optimization parameters for ultrasound-assisted extraction

Ultrasonic extraction of *Cordyceps* polysaccharides is like playing a precise sound game-the combination of power and frequency is particularly important. Usually, when the power is adjusted to around 300 W and combined with a 20 kHz sound wave frequency, the effect is the best. At this time, the cavitation bubbles produced are both dense and violent, and can tear the cell wall just right. But time control is the real technical job. The laboratory found that 15-20 minutes is a sweet spot. If it is too short, it cannot be fully extracted. If it exceeds half an hour, the polysaccharide chain begins to break. The temperature should be controlled within the range of 50 ℃-60 ℃ (Li et al., 2011), which can maintain the solvent activity without reducing the cavitation effect. Interestingly, recently, someone attempted to introduce inert gas during ultrasound and found that it could produce more stable cavitation bubbles, with the yield increasing by about 8%. However, this set of operations has higher requirements for equipment and is still at the laboratory stage at present.

The selection of solvents is actually quite mysterious-although water extraction and alcohol precipitation are classic, when different concentrations of ethanol are used for precipitation, the molecular weight distribution of polysaccharides will change significantly (Figure 1). In the laboratory, it was tested that the polysaccharide side chains obtained by precipitation with 40% ethanol were particularly numerous, while the polysaccharides obtained with 80% ethanol had larger molecular weights but a more "rigid" structure. The solid-liquid ratio is even more an art. The range of 1:15 to 1:20 has the best effect (Yan and Wang, 2006). If it is too dilute, the solvent will be wasted; if it is too thick, the extraction efficiency will decrease instead. Interestingly, last year a team added some ionic liquid to water and found that certain specific anions could form hydrogen bonds with polysaccharides, and the extraction rate soared directly to 92%. However, when it comes to industrial production, the issue of solvent recovery still needs to be taken into account. Therefore, the mainstream method for extraction at present is still using warm water, after all, the cost is there. The latest research has found that when the pH is adjusted to weakly alkaline (7.5-8.0), the yield of polysaccharides can increase by another 5-8%, possibly because the cell wall is more prone to swelling under alkaline conditions.

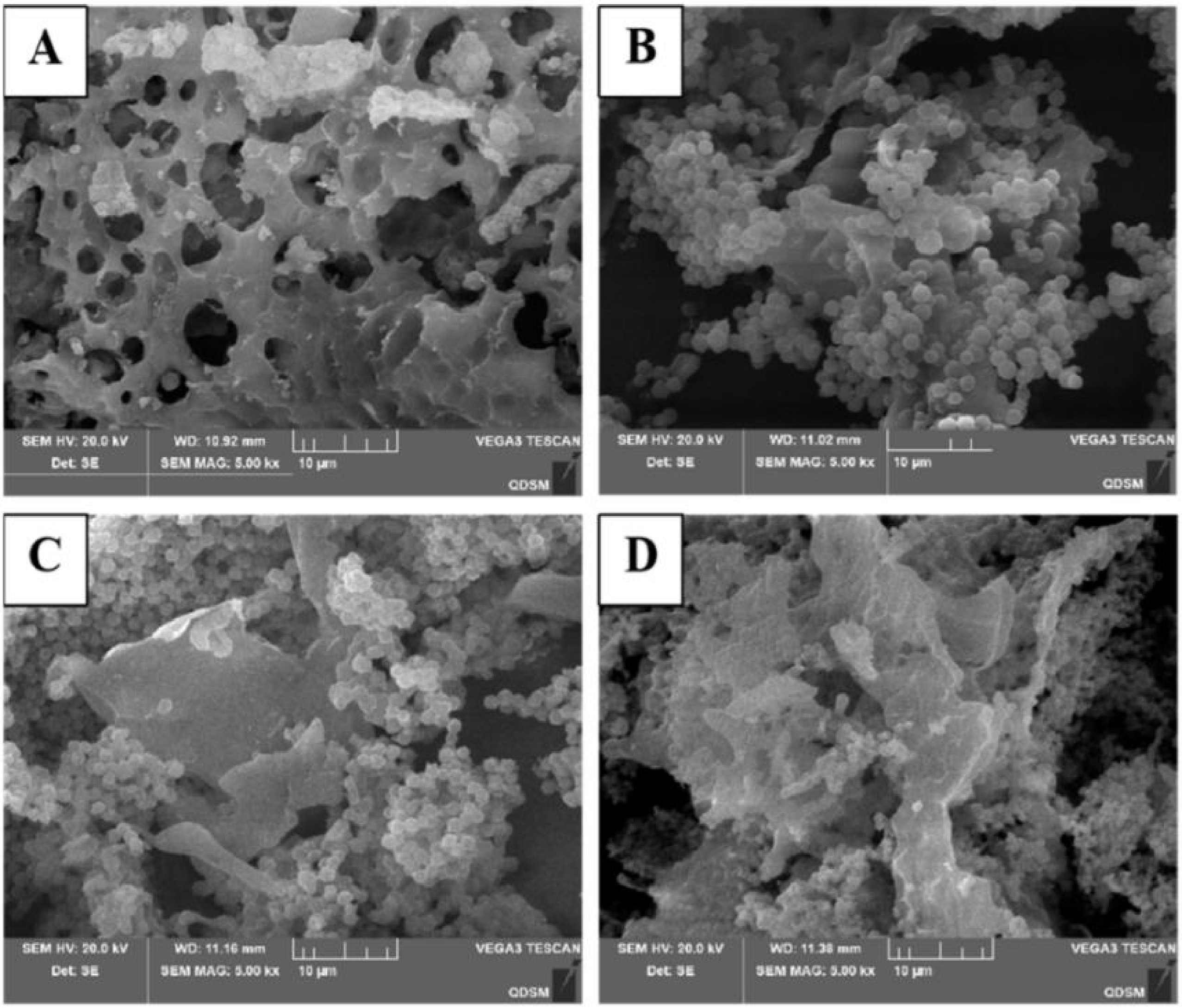

Figure 1 SEM images of CMMP−1 (A), CMMP−2 (B), CMMP−3 (C), and CMMP−4 (D) (5000×) (Adapted from Wang et al., 2024b)

Image caption: CMMP−1, CMMP−2, CMMP−3, and CMMP−4 represent four polysaccharide components obtained from ethanol solid media with different ethanol concentrations (Adapted from Wang et al., 2024b)

4.3 Advantages and challenges of ultrasound-assisted extraction

Ultrasonic extraction of *Cordyceps* polysaccharides is indeed a clever method-it can be completed in 15 minutes at the fastest, which is much more time-saving and labor-saving than the traditional hot water extraction. Laboratory data shows that the solvent usage can be reduced by about 40% (Wu et al., 2013), which is quite helpful in easing environmental protection pressure. This is more obvious in industrial applications. One set of ultrasonic equipment can replace the production capacity of three sets of traditional extraction tanks. However, this matter should be handled with caution. We encountered this situation when conducting experiments before. When the power was set above 400 W, the immune activity of polysaccharides was directly reduced by 30%. It was later discovered that the free radicals produced by ultrasonic cavitation cut the polysaccharide chains, and now the power density has to be strictly controlled. Recently, I've figured out a little trick: using intermittent ultrasonic mode (working for 2 seconds and stopping for 1 second) can not only ensure the extraction efficiency but also reduce the damage to the polysaccharide structure by 20%. Of course, when it comes to *Cordyceps sinensis* raw materials from different origins, these parameters still need to be fine-tuned. After all, the thickness of the cell walls varies quite a lot.

5 Synergistic Extraction of *Cordyceps* Polysaccharides Using Enzymatic and Ultrasound Techniques

5.1 Mechanism of synergistic extraction

When it comes to extracting *Cordyceps* polysaccharides, ultrasonic technology has indeed been of great help. Just think about it. Those stubborn cell walls usually wrap up polysaccharides tightly, but when ultrasound comes, it's like a miniature blasting hand, directly blowing up many openings in the cell walls. Now it's great. The enzymes that were originally spinning around outside can now break in and have a close contact with the substrate. However, it should be noted that the ultrasonic power should not be set too high; otherwise, even the polysaccharide structure may be damaged by vibration. Experimental data show that after appropriate ultrasonic treatment, the yield of polysaccharides can be increased by about 30%, but the specific effect still depends on the freshness of the raw materials-*Cordyceps* that has been stored for a long time will have more difficult cell walls to deal with. Interestingly, the cavitation bubbles generated by ultrasound can not only physically break the cell walls but also seem to activate certain enzymes. This discovery is quite surprising.

Combining ultrasound and enzymatic methods to extract polysaccharides yields surprisingly good results-these two working together are much better than going it alone. Ultrasound first shakes the cell walls to pieces (as can be seen from Figure 2, the cell surfaces are all wrinkled). At this point, enzymes can take advantage of the situation and accelerate the decomposition as if they have been empowered. Especially for those difficult cellulose, with the help of ultrasound, the working efficiency of cellulase doubles directly. However, it should be noted that this combination of punches should not be too forceful. If the ultrasound time exceeds 20 minutes, it will instead inactivate the enzymes. The laboratory data are quite interesting. When used in combination, the yield of polysaccharides is 40% higher than when used alone by enzymatic method (Cheung and Wu, 2013), but the cost also has to increase by 30% accordingly. Recently, it has also been found that ultrasonic treatment followed by enzyme addition yields better results than simultaneous use. This might be because the microflow generated by ultrasonic treatment makes the enzyme distribution more uniform. Of course, when it comes to different batches of *Cordyceps*, the proportion of this combination still needs to be flexibly adjusted.

5.2 Optimization parameters for synergistic extraction

When it comes to extracting polysaccharides from *Cordyceps sinensis*, the control of time and pace is particularly important. The ultrasound time is too short. The cell wall can't be broken. If it is too long, it is easy to shatter the polysaccharides. Enzymatic hydrolysis time is also a technical task-insufficient reaction leads to low yield, but if it is delayed for too long, the enzyme itself will be inactivated first. Various combinations were tried in the laboratory and it was found that the most ideal effect was achieved by first conducting a 15-minute ultrasonic "breakthrough" followed by a 2-hour enzymatic "sweep". However, this formula is not a panacea. Last time when I changed to a batch of *Cordyceps sinensis* produced in Yunnan, I had to set the ultrasound to 20 minutes to be sufficient. Interestingly, adding a 5-minute ultrasound in the middle of enzymatic hydrolysis can increase the yield by another 5%, but the operation is too troublesome and industrial production may not be able to handle it.

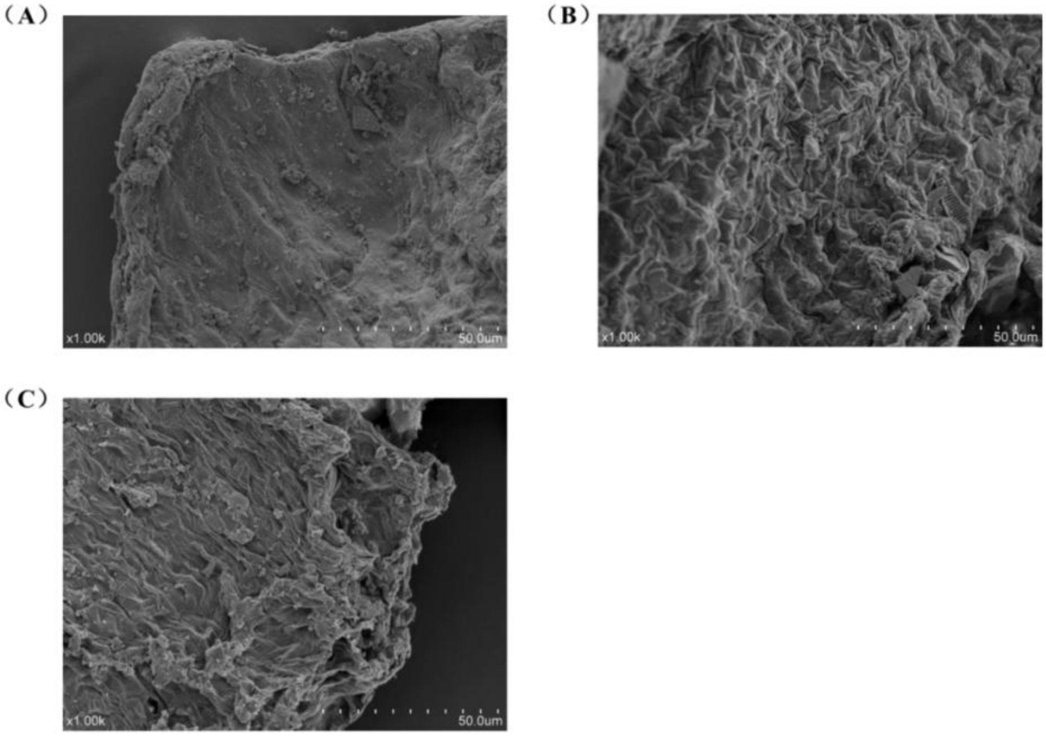

Figure 2 SEM micrographs of the surface structure of U. lactuca samples (1000×) (Adopted from Wang et al., 2024a)
Image caption: (A) untreated, (B) ultrasound-assisted hot water treatment and (C) ultrasound-assisted cellulase treatment (Adopted from Wang et al., 2024a)

When conducting ultrasound-enzymatic co-extraction, temperature and pH are two parameters that people both love and hate. Enzymes are particularly delicate-they slack off when the temperature drops to 40℃ and stop working directly when it exceeds 60℃. The pH value is even more picky. A difference of 0.5 May cause the activity to drop by half (Yang et al., 2017). But interestingly, ultrasound works best at around 55℃, which is also the "comfort zone" of most enzymes. Repeated debugging in the laboratory found that when the pH was controlled at 5.5 and the temperature was maintained in the sweet spot range of 50-55℃, the yield of polysaccharides could be increased by 25% compared with the conventional conditions. However, in actual operation, the temperature of the reaction system will continuously rise with the ultrasonic waves, so a sensitive temperature control system needs to be equipped for real-time regulation. Recently, it was also discovered that the optimal pH for *Cordyceps sinensis* from different origins actually varies slightly. This finding requires us to re-optimize our extraction process.

5.3 Advantages of synergistic extraction

The combination of ultrasound and enzymatic methods for extracting polysaccharides is indeed much more effective than doing it alone. Let's start with the output. The combined use can extract 20% to 30% more polysaccharides. This has been verified several times in the laboratory. What is even more remarkable is that the polysaccharides proposed in this way are particularly "clean" with far fewer impurities. However, the most surprising thing is that the structure of the polysaccharides was hardly damaged-those key active groups were all well preserved. Of course, in actual operation, it should be noted that the ultrasonic power should not be too high; otherwise, it may instead shatter the polysaccharide molecules. Recently, it has also been found that ultrasonic treatment followed by enzyme addition yields better results than the reverse operation. This might be because the enzymes are more likely to function when the cell walls are loosened by vibration. Although the equipment investment is a bit high, considering that the purification process can be simplified, it is still quite cost-effective in the long run.

Nowadays, when extracting polysaccharides from *Cordyceps*, environmental protection indicators are becoming increasingly important. The traditional methods consume both electricity and water. However, the ultrasonic-enzymatic synergistic extraction does have advantages in this regard-energy consumption can be reduced by about 40% (Yan and Wang, 2006), and the amount of solvent used is also halved. To be honest, the price of enzyme preparations is still a bit high. Fortunately, they can be recycled 3 to 4 times now, which greatly reduces the cost pressure. Laboratory data show that this green process not only saves resources, but also yields higher polysaccharide activity in the end. Of course, when industrial-scale amplification is carried out, the issue of equipment compatibility also needs to be addressed. After all, ultrasonic probes tend to wear out over time. Recently, some manufacturers have launched a new type of variable-frequency ultrasonic equipment. It is said to be both energy-saving and durable, but it is unknown how the actual production effect will be. Overall, this approach strikes a balance between efficiency and environmental protection. Although the initial investment is a bit larger, it is very cost-effective in the long run.

6 Performance Evaluation of Enzymatic and Ultrasound-Assisted Extraction

6.1 Extraction efficiency and polysaccharide purity

Recently, it was discovered that the combination of ultrasound and enzymatic methods for extracting polysaccharides from *Cordyceps sinensis* surprisingly works well. Laboratory data show that when dealing with mycelial medium, this combination can achieve a yield of 15.43%, which is approximately 2 percentage points higher than using the enzymatic method alone. However, microwave extraction is said to have performed more vigorously (Zhu et al., 2016), but unfortunately the specific data have been concealed and not fully disclosed. Interestingly, even with the assistance of ultrasound, the effect of processing mycelium in liquid culture is significantly worse, which may be related to the difference in cell wall structure. These new methods are indeed much better than the old-fashioned hot water extraction, but the equipment investment cost will also increase accordingly. Small-scale production really needs to think twice.

Purifying *Cordyceps* polysaccharides is by no means an easy task. Take Sephadex G-100 column chromatography as an example. Although polysaccharides can be separated by molecular weight, interestingly, polysaccharides composed of the same monosaccharides still have differences in properties. The polysaccharides extracted by ultrasound are quite special. The molecular arrangement is tighter than that obtained by other methods, which might be due to the fact that ultrasound has shaken off some impurity proteins. However, during the purification process, it was found that some small-molecule impurities were particularly stubborn and could not be dealt with merely by gel chromatography. In the laboratory, it was tried that by combining ultrasonic extraction with ion-exchange chromatography, the purity could be increased from 75% to around 90%. However, with more steps, the cost also rose accordingly.

6.2 Bioactivity evaluation

The antioxidant capacity of *Cordyceps* polysaccharides is indeed quite remarkable, especially CMMP-1 obtained by ultrasound-assisted enzymatic method. The experimental data are quite impressive. Ultrasonic extraction alone is not bad either, and it is very effective against ABTS and hydroxyl radicals (Xiao et al., 2012). However, when it comes to immune regulation, although no direct macrophage experimental data have been observed, there is an interesting phenomenon-polysaccharides extracted by microwave have the best anti-tumor effect, which might also be related to immune regulation. It was also found in the laboratory that although polysaccharides obtained by different extraction methods all have antioxidant properties, the efficiency of eliminating different free radicals varies greatly. This might be related to their molecular configurations. Unfortunately, nowadays, few *Cordyceps* products on the market indicate the specific extraction process, and consumers have no idea at all about the activity of the polysaccharides they purchase.

6.3 Economic and environmental benefit analysis

Nowadays, the extraction of *Cordyceps* polysaccharides has indeed brought many benefits through new technologies. Enzymatic and ultrasound-assisted methods not only save electricity and water, but more importantly, the polysaccharides extracted have particularly high activity, which is just right for making health products. After optimization by Response Surface Analysis (RSM), the production efficiency can be increased by another 20% to 30%. Although the initial equipment investment is a bit large, it is quite cost-effective in the long run. The advantages in environmental protection are more obvious-traditional methods often require the use of strong acids and strong alkalis, while the new process basically does not use these hazardous chemicals, and the carbon footprint is significantly reduced. However, in actual operation, it was found that the raw materials of different batches vary greatly. Even the parameters optimized by RSM sometimes need to be fine-tuned. Recently, some manufacturers have attempted to turn the extracted waste residue into organic fertilizer, which can be regarded as maximizing the utilization of resources. However, whether this by-product can be profitable remains to be seen.

7 Case Studies on Enzymatic and Ultrasound-Assisted Extraction

7.1 Laboratory-scale cases

Recently, those who are fiddling with the extraction of *Cordyceps* polysaccharides in the laboratory have all been fond of the combination of ultrasonic and enzymatic methods. There was a rather interesting experiment. After messing around with the parameters using the Response surface method (RSM) for a long time, a yield of 15.43% was finally achieved-this figure is quite impressive among peers. However, the selection of proteases also requires careful consideration. Alkaline proteases are more powerful than others, reaching up to 13.01% (Li and Liu, 2015), although they are not as effective as the combination of ultrasound. In fact, these small-scale trial data look good, but scaling up production is another matter. Last year, a factory conducted a pilot test based on the parameters of a paper and directly reduced the yield by 20%. Later, it was found that the purity of the industrial-grade enzyme preparation was insufficient. Now everyone is gradually getting the upper hand. Laboratory data needs to be given a headshot before it can be moved to the production line. After all, a 50-liter reactor and a 50-milliliter conical flask are completely different things. By the way, the polysaccharides extracted by these new methods are indeed highly active, and there should be a considerable market for high-end health products.

7.2 Pilot-scale validation

In fact, conducting ultrasonic extraction of *Cordyceps* polysaccharides on a pilot-scale is much more troublesome than in a laboratory-after the equipment is scaled up, all parameters have to be readjust. There was an interesting study. After fidling with the random center optimization method for a long time, the yield was finally raised to 16.6% in a 50-liter reactor, which was a little higher than the laboratory data. However, in actual operation, it was found that the power stability of industrial-grade ultrasonic generators is far worse than that of laboratory equipment, and it is necessary to keep a close eye on the instruments for adjustment at all times. The most troublesome issue was the heat dissipation problem after amplification. The negligible thermal effect in the small tank could directly raise the temperature of the material liquid to 70 degrees after amplification. Later, a cooling system was added to solve the problem. Although these pilot-scale experiences are quite troublesome, they do prove that ultrasonic technology can be applied to industrial use. However, for each specific factory, the process may still need to be fine-tuned according to their own equipment. Now it seems that this method should have no problem running on a 5-ton tank. If it is scaled up further, it may require re-exploration.

7.3 Industrial application prospects

Nowadays, many enterprises are thinking about how to transfer the ultrasonic-enzymatic process to the production line. To be honest, *Cordyceps* polysaccharides are becoming increasingly popular in the health supplement circle, especially for those antioxidant and immunomodulative functions (Zhu et al., 2016), but traditional extraction methods really can't keep up with market demand. The pilot-scale data is quite impressive, proving that this new process can also work well in scale-up production. However, for real industrialization, many practical problems still need to be solved-such as the layout of ultrasonic probes in large tanks, or how to recycle those expensive enzyme preparations. Recently, a company from Fujian Province has been in trial production and found that controlling the ultrasonic power in different zones can save 20% of energy consumption. Although the cost of equipment transformation is a bit high, it is quite cost-effective in the long run. Both domestic and foreign peers are keeping a close eye on this matter. After all, whoever can master the process first will be able to seize the initiative in the next-generation functional food market. However, to be precise, the biggest bottleneck at present is actually that the industry standards have not yet kept up. The activity of polysaccharides produced by the same process from different manufacturers can vary by several tens of percent. This needs to be standardized as soon as possible.

8 Challenges and Future Directions for Green Extraction Technologies

8.1 Bottlenecks in technology promotion

When it comes to green extraction technology, enterprises have a love-hate relationship with it. Equipment investment is indeed a hurdle-a set of industrial-grade ultrasonic equipment often costs over a million yuan, and enzyme preparations are even more costly, which small factories simply cannot afford. Different *Cordyceps* varieties are particularly sensitive. For example, the extraction parameters of *Cordyceps militaris* and *Cordyceps sinensis* can differ by 20%, and just the process adjustment can drive the R&D team crazy. Last year, a company purchased imported equipment, only to find that it was particularly poorly compatible with local *Cordyceps sinensis* raw materials, and had to spend extra money to modify it. What's more troublesome is that there is not even a standard process in the industry at present. The effect of the same method produced in different factories is unstable (Mena-Garcia et al., 2019). However, the recently emerged technology service companies do offer process customization, helping enterprises save a lot of trial-and-error costs, but the service fees are still not cheap. Ultimately, for these new technologies to be popularized, it will have to wait for the localization of equipment and the implementation of industry standards.

8.2 Future research directions in green extraction

What should be the next step for the extraction technology of *Cordyceps* polysaccharides? Nowadays, researchers are all considering a combination of green technologies-the mixed use of microwave, ultrasound and enzymatic methods. Experiments have long shown that after microwave pretreatment followed by ultrasonic-enzymatic method, the extraction efficiency can increase by another 15%, but equipment compatibility is a new challenge (Wen et al., 2020). Recently, it has become more fashionable to have AI act as an experimenter and predict the optimal process parameters through algorithms (Wang, 2024), which is said to save 60% of the debugging time (Hou et al., 2024). But to be honest, for these high-end technologies to be implemented, they still have to overcome the cost barrier. After all, enterprises are not willing to pay for the beautiful data in the laboratory. Although ultrasonic and enzymatic methods are environmentally friendly, when industrially scaled up, either the probe life is short or the recovery rate of enzyme preparations is low (Ahmed et al., 2024). Interestingly, some teams have attempted to use discarded agricultural raw materials as enzyme culture media, which not only reduces costs but is also environmentally friendly. However, the stability of enzyme activity is still a bit lacking. The breakthrough point in the next five years is likely to focus on how to make these technologies both maintain the green advantage and be accounted for by bosses (Yin et al., 2018).

8.3 Development of a sustainable industrial chain

For the *Cordyceps sinensis* industry to truly achieve a green transformation, merely improving the extraction technology is far from enough. We need to focus on the entire production chain-can those used ethanol solvents be recycled? How to deal with the discarded mushroom residue? These problems are becoming increasingly urgent now (Li et al., 2017). Some manufacturers have attempted to establish a closed-loop circulation system to recycle and reuse 95% of the extracted solvents. Although the initial investment is large, it is both cost-effective and environmentally friendly in the long run (Wang et al., 2017). However, to achieve these, it is not enough to rely solely on enterprises. Research institutions need to provide technical support, and government departments should introduce supporting policies, such as offering tax incentives for green processes. Recently, industry associations have been formulating carbon footprint standards for *Cordyceps* production. This is quite important. After all, only when standards are in place can everyone know in which direction to strive. Ultimately, sustainable development is not a matter of a single link, but a revolution covering the entire chain from cultivation to extraction and then to waste disposal. Without any one of these links, it cannot be accomplished.

Acknowledgments

Thank you to the reviewers for their thorough and detailed reading of this manuscript and for providing valuable suggestions for improvement.

Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Ahmed T., Suzauddula M., Akter K., Hossen M., and Islam M., 2024, Green technology for fungal protein extraction—a review, Separations, 11(6): 186. https://doi.org/10.3390/separations11060186

Cheong K., Wang L., Wu D., Hu D., Zhao J., and Li S., 2016, Microwave-assisted extraction, chemical structures, and chain conformation of polysaccharides from a novel *Cordyceps sinensis* fungus UM01, Journal of Food Science, 81(9): C2167-C2174. https://doi.org/10.1111/1750-3841.13407

Cheung Y., and Wu J., 2013, Kinetic models and process parameters for ultrasound-assisted extraction of water-soluble components and polysaccharides from a medicinal fungus, Biochemical Engineering Journal, 79: 214-220. https://doi.org/10.1016/J.BEJ.2013.08.009

Cheung Y., Liu X., Wang W., and Wu J., 2015, Ultrasonic disruption of fungal mycelia for efficient recovery of polysaccharide-protein complexes from viscous fermentation broth of a medicinal fungus, Ultrasonics Sonochemistry, 22: 243-248. https://doi.org/10.1016/j.ultsonch.2014.05.006

Gao N., Zhang W., Hu D., Lin G., Wang J., Xue F., Wang Q., Zhao H., Dou X., and Zhang L., 2023, Study on extraction, physicochemical properties, and bacterio-static activity of polysaccharides from *Phellinus linteus*, Molecules, 28(13): 5102. https://doi.org/10.3390/molecules28135102

Guan J., Yang F., and Li S., 2010, Evaluation of carbohydrates in natural and cultured *Cordyceps* by pressurized liquid extraction and gas chromatography coupled with mass spectrometry, Molecules, 15(6): 4227-4241. https://doi.org/10.3390/molecules15064227

Hou F., Song S., Yang S., Wang Y., Jia F., and Wang W., 2024, Study on the optimization, extraction kinetics and thermodynamics of the ultrasound-assisted enzymatic extraction of *Tremella fuciformis* polysaccharides, Foods, 13(9): 1408. https://doi.org/10.3390/foods13091408

Li W.F., and Liu J.M., 2015, Study on optimization of enzymatic extraction of polysaccharide from *Cordyceps militaris* with ultrasonic-assisted by response surface methodology, Guiding Journal of Traditional Chinese Medicine and Pharmacy, 21(20): 24-27, 30.

Li Y.H., Song H., and Li H.Y., 2011, Optimization of ultrasound-assisted extraction of polysaccharides from *Cordyceps* flowers, Journal of Li-shizhen Traditional Chinese, 22(6): 1456-1458.

Li Z., Chen A., Li Z., Qu M., Chen H., Yang B., and Wang Y., 2017, A novel and environmentally friendly bioprocess for separation and partial purification of polysaccharides from *Cordyceps sinensis* mycelia by an aqueous two-phase system, RSC Advances, 7(60): 37659-37665. https://doi.org/10.1039/C7RA05360F

Liu X., Zhu Z., Tang Y., Wang M., Wang Z., Liu A., and Zhang Y., 2016, Structural properties of polysaccharides from cultivated fruit bodies and mycelium of *Cordyceps militaris*, Carbohydrate Polymers, 142: 63-72. https://doi.org/10.1016/j.carbpol.2016.01.040

Luo X., Duan Y., Yang W., Zhang H., Li C., and Zhang J., 2017, Structural elucidation and immunostimulatory activity of polysaccharide isolated by subcritical water extraction from *Cordyceps militaris*, Carbohydrate Polymers, 157: 794-802. https://doi.org/10.1016/j.carbpol.2016.10.066

Mena-García A., Ruiz-Matute A., Soria A., and Sanz M., 2019, Green techniques for extraction of bioactive carbohydrates, TrAC Trends in Analytical Chemistry, 119: 115612. https://doi.org/10.1016/J.TRAC.2019.07.023

Nadar S., Rao P., and Rathod V., 2018, Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: a review, Food Research International, 108: 309-330. https://doi.org/10.1016/j.foodres.2018.03.006

Usman M., Nakagawa M., and Cheng S., 2023, Emerging trends in green extraction techniques for bioactive natural products, Processes, 11(12): 3444. https://doi.org/10.3390/pr11123444

Wang J., Nie S., Chen S., Phillips A., Phillips G., Li Y., Xie M., and Cui S., 2017, Structural characterization of an α-1, 6-linked galactomannan from natural *Cordyceps sinensis*, Food Hydrocolloids, 78: 77-91. https://doi.org/10.1016/J.FOODHYD.2017.07.024

Wang L., Li T., Liu F., Liu D., Xu Y., Yang Y., Zhao Y., and Wei H., 2019, Ultrasonic-assisted enzymatic extraction and characterization of polysaccharides from dandelion (*Taraxacum officinale*) leaves, International Journal of Biological Macromolecules, 126: 846-856. https://doi.org/10.1016/j.ijbiomac.2018.12.232

Wang W., 2024, AI based drug screening process: from data mining to candidate drug validation, Bioscience Method, 14(1): 37-49. https://doi.org/10.5376/bm.2024.15.0005

Wang W., and Huang Q.K., 2024, The application prospects of artificial intelligence in molecular medicine, International Journal of Molecular Medical Science, 14(1): 16-23. https://doi.org/10.5376/ijmms.2024.14.0003

Wang W., Li J., Lu F., and Liu F., 2024a, Ultrasound-assisted multi-enzyme extraction for highly efficient extraction of polysaccharides from *Ulva lactuca*, Foods, 13(6): 891.
https://doi.org/10.3390/foods13060891

Wang X., Zhang J., Zhang K., Guo Z., Xu G., Huang L., Wang L., and Li J., 2024b, Ultrasound-assisted enzyme extraction, physicochemical properties and antioxidant activity of polysaccharides from *Cordyceps militaris* solid medium, Molecules, 29(19): 4560.
https://doi.org/10.3390/molecules29194560

Wen L., Zhang Z., Sun D., Sivagnanam S., and Tiwari B., 2020, Combination of emerging technologies for the extraction of bioactive compounds, Critical Reviews in Food Science and Nutrition, 60(11): 1826-1841.
https://doi.org/10.1080/10408398.2019.1602823

Wu Q.Y., Chen L., Qu H.S., Jia J.Q., Yan H., and Gui Z.Z., 2013, Process optimization of ultrasound-assisted polysaccharide extraction from the fruiting bodies of silkworm *Cordyceps militaris*, Acta Sericologica Sinica, 39(3): 587-592.

Xiao J., Sun J., Yao L., Zhao Q., Wang L., Wang X., Yuan X., and Zhao B., 2012, Physicochemical characteristics of ultrasonic extracted polysaccharides from cordyceps cephalosporium mycelia, International journal of biological macromolecules, 51(1-2): 64-69.
https://doi.org/10.1016/j.ijbiomac.2012.04.029

Xu L., Guo S., Li Y., Guo W., Guo X., and Hong S., 2023, Ultrasound-assisted enzymatic extraction and bioactivity analysis of polypeptides from *Cordyceps militaris*, Journal of Chemistry, 2023(1): 1233867.
https://doi.org/10.1155/2023/1233867

Yan J., and Wang Y., 2006, Random-centroid optimization of ultrasound-assisted extraction of polysaccharides from *Cordyceps sinensis* mycelium, Current Topics in Nutraceuticals Research, 13(4): 167.

Yan J., Wang W., and Wu J., 2013, Recent advances in *Cordyceps sinensis* polysaccharides: mycelial fermentation, isolation, structure, and bioactivities: a review, Journal of Functional Foods, 6: 33-47.

Yang H., Wu Z., He D., Zhou H., and Yang H., 2017, Enzyme-assisted extraction and Pb^{2+} biosorption of polysaccharide from *Cordyceps militaris*, Journal of Polymers and the Environment, 25(4): 1033-1043.
https://doi.org/10.1007/s10924-016-0882-4

Yin C., Fan X., Fan Z., Shi D., and Gao H., 2018, Optimization of enzymes-microwave-ultrasound assisted extraction of *Lentinus edodes* polysaccharides and determination of its antioxidant activity, International Journal of Biological Macromolecules, 111: 446-454.
https://doi.org/10.1016/j.ijbiomac.2018.01.007

Zhu Z., Dong F., Liu X., Lv Q., Ma Y., Liu F., Chen L., Wang T., Wang Z., and Zhang Y., 2016, Effects of extraction methods on the yield, chemical structure and anti-tumor activity of polysaccharides from *Cordyceps gunnii* mycelia, Carbohydrate Polymers, 140: 461-471.
https://doi.org/10.1016/j.carbpol.2015.12.053