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Investigation of the effects of pulsed ultrasound in aerated submerged culture of *Phellinus linteus* for biomass and polysaccharide production

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Abstract. *Phellinus linteus* is a valuable medicinal mushroom widely used in Japan and Korea, particularly for its immune-boosting and anti-tumor properties. It contains a range of chemical components, including amino acids, vitamins, minerals, carbohydrates, and bioactive compounds such as polysaccharides, protein-polysaccharides, steroids, terpenoids, and flavonoids. In Vietnam, this mushroom has become rare and costly due to its long growth cycle and overharvesting from the wild. To address this issue, submerged mycelial culture has emerged as a promising solution to reduce cultivation time while yielding extracts rich in bioactive compounds. This study explores the effect of pulsed ultrasound on the mycelial growth and polysaccharide synthesis of *Phellinus linteus* from Ba Be District, Bac Kan Province, Vietnam (designated as P.B91). Optimal conditions for culturing P.B91 were identified using ultrasound at a frequency of 20 kHz and an intensity of 12 W/cm² for 150 seconds, applied 108 hours post-inoculation. Ultrasonic treatment resulted in biomass yield and intracellular and extracellular polysaccharide levels of 20.73 g/L (33.96 g/L in post-culture medium), 7.14 g/L, and 5.10 g/L, respectively, representing increases of 3.34, 4.25, and 3.88 times compared to the control. These findings demonstrate the potential of ultrasonic waves in submerged culture to improve quality, reduce cultivation time, and enhance energy efficiency over traditional aerated submerged culture methods.

Keywords: Biomass, *Phellinus linteus*, Polysaccharides, submerged culture, Ultrasonic

Classification numbers: 1.1,

1. INTRODUCTION

Phellinus linteus is a well-known medicinal mushroom with high pharmacological value, rich in polysaccharides, terpenoids, steroids, and flavonoids. It exhibits various pharmacological effects, such as improving blood circulation, preventing and treating heart disease, enhancing detoxification, protecting the liver, and offering anti-allergic, anti-diabetic, and stress-relieving properties. Notably, it has superior anti-tumor properties compared to other medicinal mushroom species [1]. Currently, the global yield of *Phellinus* species is only around 30 tons per year, mostly harvested from the wild. Due to its multi-year life cycle and long growing period, *Phellinus linteus* has become scarce and expensive in the wild as human harvesting depletes natural resources. As a result, artificial cultivation has emerged as an effective way to obtain the active compounds of *Phellinus linteus*. However, current cultivation methods face challenges such as a long production cycle, low yield, and high costs. To address these challenges, submerged fermentation, coupled with aeration, has emerged as a promising approach to shorten cultivation times and enhance biomass accumulation. This method has the potential to significantly increase the content of bioactive compounds.

In addition, ultrasound technology has gained popularity in various fields such as food processing, fermentation, medical technology, and chemical engineering. Low-frequency ultrasound, in particular, is known to accelerate chemical reactions, support microbial fermentation, promote cell growth, and improve product quality in the food industry. The mechanical effects of ultrasound can induce cavitation, mechanical, and thermal impacts that alter cell membrane permeability, increase cell growth rates, modify molecular structures, and enhance reaction processes. Additionally, it activates intracellular signal transduction systems and alters the synthesis of metabolites in organisms [2]. For instance, Pitt et al. (2003) suggested that ultrasonic treatment can enhance the transport of oxygen and nutrients essential for microbial cell growth, while also reducing feedback inhibition, potentially accelerating the growth of microorganisms [3].

Several studies have demonstrated the positive impact of ultrasound on fungal biomass production and metabolite synthesis. For example, under optimal ultrasound conditions, the biomass of *Ganoderma lucidum* mycelia increased by 26.99 %, and triterpenoid synthesis increased by 33.62 %. Ultrasound also significantly boosted both intracellular and extracellular polysaccharide levels by 18.48 % and 35.90 %, respectively, and increased flavonoid content by 30.27 % and 2.00 %, compared to control groups [4]. In recent years, considerable progress has been made in optimizing the growth and metabolism of *Phellinus linteus* through modifications to culture media and fermentation parameters. This study explores the application of ultrasonic treatment during the submerged culture of the P.B91 strain to enhance both mycelial biomass production and polysaccharide content.

2. MATERIALS AND METHODS

2.1. Materials

The *Phellinus linteus* strain, labeled P.B91, was collected from a natural reserve in Ba Be district, Bac Kan province, Vietnam. It was subsequently preserved at 4 °C on PGA medium at the Vietnam Institute of Agricultural Engineering and Postharvest Technology.

Chemicals: Fructose were from Applichem (Darmstadt, Germany), KH_2PO_4, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $FeCl_2 \cdot 6H_2O$, and $CuSO_4 \cdot 7H_2O$ were purchased from Merck

(Darmstadt, Germany). All chemicals used in this study were of analytical grade, suitable for chemical analysis, and met the required quality standards.

Ultrasonic device: The ultrasonic treatment was performed using a TJS-3000 intelligent Ultrasonic Generator V6.0 (Hangzhou Success Ultrasonic Equipment Co., Ltd), operating at a frequency of 20 kHz and a maximum power output of ≤3000 W. The power output was variable and adjustable according to the specific needs of the application.

2.2. Methods

2.2.1. Breeding

The P.B91 strain was preserved on PGA medium (composed of 200 g potato, 20 g glucose, 20 g agar, and 1000 mL distilled water) and stored at 4°C. Prior to experimentation, the P.B91 strain was reactivated by inoculating it into a 500 mL flask containing 200 mL of LM medium. The culture was incubated at 28°C on a rotary shaker at 120 rpm for 7 days.

The LM liquid medium was prepared according to *Lee et al.* [5], and contained the following components: yeast extract, 20 g/L; fructose, 40 g/L; KH_2PO_4, 1.00 g/L; $MgSO_4 \cdot 7H_2O$, 0.50 g/L; K_2HPO_4, 0.46 g/L; $MnCl_2 \cdot 4H_2O$, 0.036 g/L; $ZnCl_2$, 0.03 g/L; $FeCl_2 \cdot 6H_2O$, 0.01 g/L; and $CuSO_4 \cdot 7H_2O$, 0.005 g/L. The initial pH of the medium was adjusted to 5.5.

2.2.2. Culture Conditions

The P.B91 fungus was cultured in fermentation tanks, each containing 30 liters of LM medium. The inoculum ratio of P.B91 was set at 10^6 CFU/g (colony-forming units per gram), with fermentation conditions maintained at 28 ± 2 °C, an aeration rate of 0.6 vvm (volume of air per volume of liquid per minute), and a stirring speed of 100 rpm [5].

2.2.3 Effect of Ultrasonic intensity in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

Following the conditions outlined in Section 2.2.2, after 96 hours of incubation, each experimental setup was subjected to ultrasound treatment with varying ultrasonic intensities of 0 W/cm² (control), 10 W/cm², 11 W/cm², 12 W/cm², 13 W/cm², and 14 W/cm² at a frequency of 20 kHz for 120 seconds. The culture was then continued until 120 hours, at which point the biomass and polysaccharide content were measured.

2.2.4 Effect of Ultrasonic Duration in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

Under the same conditions as in Section 2.2.2, after 96 hours of incubation, each experimental setup was subjected to ultrasound treatment with an intensity of 12 W/cm² at a frequency of 20 kHz, for varying durations of 0 seconds (control), 90 seconds, 120 seconds, 150 seconds, 180 seconds, and 210 seconds. After ultrasound treatment, the cultures were continued until 126 hours, at which point biomass and polysaccharide content were measured.

2.2.5 Effect of Ultrasonic timing in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

As described in Section 2.2.2, in each experiment, ultrasound treatment was applied at different culture times (24, 48, 60, 72, 84, 96, 108, 120, and 132 hours) at an ultrasonic intensity of 12 W/cm², a frequency of 20 kHz, and a duration of 150 seconds. After ultrasound treatment,

the cultures were continued until 132 hours, at which point biomass and polysaccharide content were measured.

2.2.6. Effect of Submerged Culture Duration of P.B91 on Biomass and Polysaccharide Production

Under the conditions described in Section 2.2.2, in each experiment, both control (no ultrasound) and ultrasound-treated setups were implemented, with ultrasonic treatment applied after 108 hours of culture (at an intensity of 12 W/cm², frequency of 20 kHz, and duration of 150 seconds). Biomass and polysaccharide content were measured at multiple time points: 90, 96, 102, 108, 114, 120, 126, 132, 138, 144, 150, 156, 162, 168, 174, 180, and 186 hours post-inoculation.

All experiments were repeated at least three times, and the results represent the average values from these replicates.

2.2.7 Monitoring Parameters

Dried Mycelial Biomass: The mushroom culture solution was collected and filtered using pre-dried filter paper to a constant weight. The mycelial biomass was then dried at 50°C until reaching constant weight [4].

Intracellular Polysaccharide Content: The dried fungal mycelial biomass was extracted with water at 90°C for 30 minutes. The polysaccharide content in the mushroom extract was determined using the phenol-sulfuric acid method [6]. Specifically, 400 µl of sample solution containing polysaccharides was mixed with 200 µl of 5% phenol solution. Then, 1 ml of concentrated $H_2 SO_4$ was added, and the mixture was allowed to sit for 30 minutes at room temperature. The resulting color was measured using a spectrophotometer at a wavelength of 490 nm. Polysaccharide content was quantified by comparing the optical density (OD) values of the sample with a glucose standard curve. The regression equation obtained was $y = 0.0117x + 0.051$, $R^2 = 0.9929$.

Extracellular Polysaccharide Content: After filtering the culture solution, the filtrate was mixed with ethanol (96 %) in a 1:4 ratio and left overnight in a refrigerator at 4 °C. The precipitated polysaccharides were then filtered using pre-dried filter paper to a constant weight. The filter paper was dried at 50 °C until constant weight to determine the polysaccharide yield.

2.2.8 Analysis of Data

All experimentswere performed in triplicate and the data were analyzed using one-way analysis of variance (ANOVA) to assess differences among mean values, with significance set at $p < 0.05$. Statistical analysis was performed using Statgraphics Plus software, version 5.1.

3. RESULTS AND DISCUSSION

3.1. Effect of Ultrasonic intensity in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

Research on optimal ultrasonic intensity is a crucial factor in enhancing biomass and polysaccharide production in *Phellinus linteus* (P.B91). Excessive ultrasonic intensity can damage cell structures or cause cell rupture, leading to macromolecular degradation or cell death. Conversely, too low an intensity may not significantly affect the mycelium. Figure 1 presents the

effects of different ultrasonic intensities on mycelial biomass and polysaccharide production in P.B91.

The results indicate that different ultrasonic intensities had statistically significant effects on mycelial biomass and polysaccharide production at the 5 % significance level. The highest yields of mycelial biomass (8.67 g/L), intracellular polysaccharides (3.58 g/L), and extracellular polysaccharides (2.84 g/L) were observed at an ultrasonic intensity of 12 W/cm². When the ultrasonic intensity was increased but kept below 13 W/cm², both mycelial biomass and metabolite synthesis increased compared to the control. However, at an intensity of 14 W/cm², the dry mycelial weight (4.12 g/L) and polysaccharide content (1.38 g/L intracellular and 1.27 g/L extracellular) decreased compared to the control.

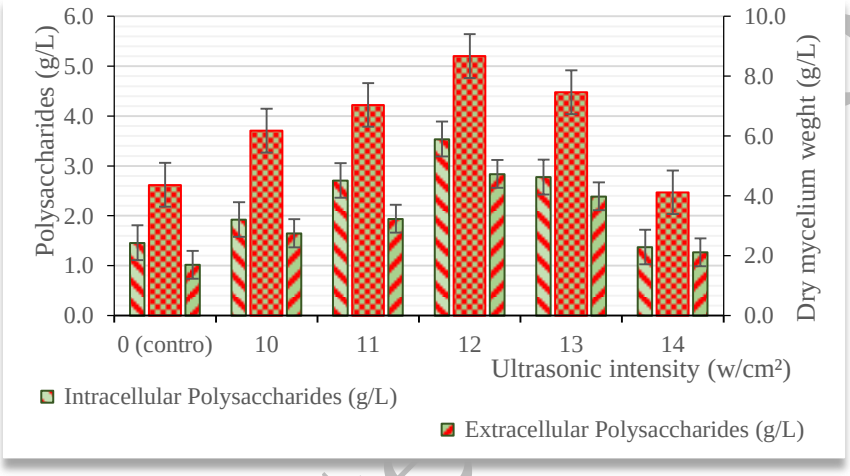

Figure 1. Effect of ultrasonic intensity in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

The gradual increase in biomass and metabolite synthesis at intensities below 13 W/cm² could be due to the beneficial effects of mild ultrasonic treatment. The energy input at these lower intensities is sufficient to stimulate mycelial growth and compound synthesis without causing cellular damage. However, higher intensities inhibited mycelial growth, likely due to the destructive effects of excessive energy on cellular structures [7]. thereby impairing the normal growth, reproduction, and metabolism of P.B91. The cavitation effect of ultrasonic waves, when applied at appropriate energy levels, can temporarily and reversibly increase cell membrane permeability, facilitating the transport of materials across the cell membrane [8]. However, prolonged or excessive cavitation may damage cell structures, thereby negatively affecting normal development and metabolism. Based on these findings, an ultrasonic intensity of 12 W/cm² was selected for further experiments.

3.2. Effect of Ultrasonic Duration in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

The effects of varying ultrasonic treatment durations on the mycelial biomass and polysaccharide production of P.B91 are presented in Figure 2.

As the ultrasonic treatment duration increased, both the biomass growth rate and polysaccharide production of P.B91 initially rose, followed by a gradual decline. Specifically, at

150 seconds of treatment, the highest levels of mycelial biomass and polysaccharide production were observed, reaching 14.19 g/L, 5.02 g/L, and 4.50 g/L, respectively ($p < 0.05$). These results suggest that the cavitation effect induced by ultrasound temporarily enhances cell membrane permeability, improving the transport of nutrients and metabolites across the cell membrane [8].

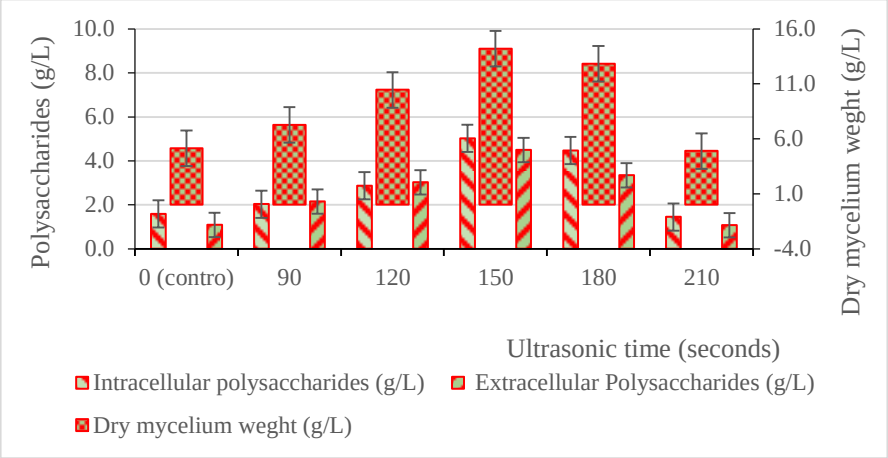

Figure 2. Effect of Ultrasonic Duration in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production

However, prolonged exposure to ultrasound beyond this point may cause excessive cavitation, leading to disruption of cell structures, cleavage of biological macromolecules, or irreversible damage to cellular components, which impairs normal growth and metabolic functions. Based on the observed trends and statistical significance, a treatment duration of 150 seconds was selected for subsequent experiments.

3.3. Effect of Ultrasonic Timing in the Submerged Culture of P.B91 on Biomass and Polysaccharide Production

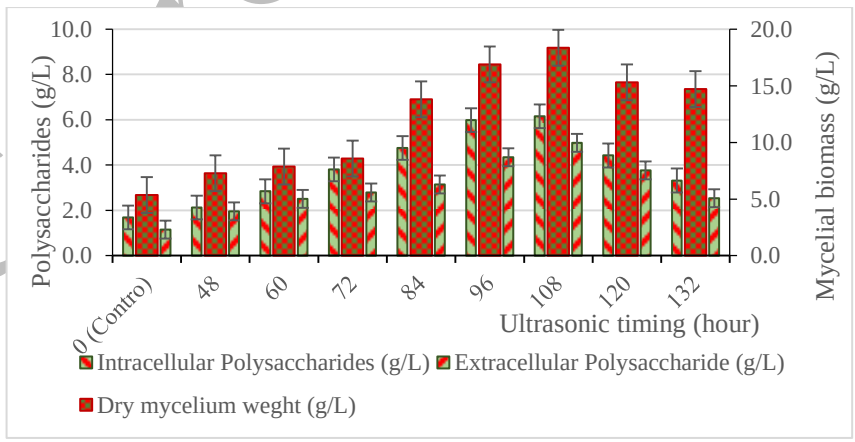

Figure 3. Effect of ultrasonic timing in the Submerged Culture of P.B91 for Biomass and Polysaccharide Production.

Choosing the optimal time for ultrasonic intervention is critical to maximizing the biomass and total polysaccharide content of P.B91. If ultrasound is applied too early, while the mycelium is still weak and not fully adapted to the culture environment, it may cause damage to the mycelium. However, when applied at the appropriate time, ultrasound can positively affect cell walls, enhance metabolism, improve mass transfer, and accelerate chemical reactions. Figure 3 illustrates the effects of ultrasonic treatment at different growth stages on mycelial biomass and both intracellular and extracellular polysaccharide content of P.B91. The results show that ultrasonic intervention during the logarithmic growth phase, specifically at 108 hours post-inoculation, significantly promoted the fermentation process of P.B91.

At 108 hours, compared to the control, the mycelial biomass increased to 18.34 g/L, and the polysaccharide content in both the mycelium and the culture medium reached 6.15 g/L and 4.98 g/L, respectively. These values were statistically significant ($p < 0.05$), with the data showing a strong improvement over the control group. In contrast, earlier intervention times (48-96 hours post-inoculation) yielded less favorable results. During these stages, mycelial cells are primarily focused on adapting to the new environment and proliferating, making them more vulnerable to damage from external stimuli such as ultrasound. As a result, ultrasonic treatment during this phase led to slower growth rates and lower biomass and polysaccharide production.

In contrast, during the logarithmic growth phase (around 108 hours), the mycelium undergoes rapid growth, robust metabolism, and exhibits increased tolerance to external stresses. As such, ultrasonic treatment at this point resulted in significantly higher biomass and polysaccharide production compared to the control group, with $p < 0.05$ for all measurements. This enhancement can be attributed to ultrasound's beneficial effects, including increased dissolved oxygen and improved permeability of the cell membrane, facilitating more efficient nutrient uptake and metabolic processes. These findings are consistent with similar studies on other fungi, such as *Ganoderma lucidum*, where ultrasound treatment also promoted higher biomass and bioactive compound production [4].

Therefore, the optimal timing for ultrasonic intervention in P.B91 was determined to be 108 hours post-inoculation, and this timing was applied in subsequent experiments.

3.4. Effect of Submerged Culture Duration of P.B91 on Biomass and Polysaccharide Production

Figure 4 shows the typical time course of mycelial growth and polysaccharide content in the aerated submerged culture of P.B91. Under aerated submerged culture conditions with ultrasonic treatment at a frequency of 20 kHz and an intensity of 12 W/cm² for 150 seconds after 108 hours of culture, the biomass, intracellular polysaccharide, and extracellular polysaccharide content of P.B91 increased rapidly during the growth phase, reaching maximum values after 138 hours. These values were 20.73 g/L for biomass (33.96 g/L in post-culture medium), 7.14 g/L for intracellular polysaccharide, and 5.10 g/L for extracellular polysaccharide. In contrast, when P.B91 was cultured under identical aerated submerged conditions without ultrasonic assistance (same inoculum ratio, pH, temperature, medium, and culture duration), the dry biomass was significantly lower at 6.21 g/L, with intracellular and extracellular polysaccharide contents also reduced to 1.84 g/L and 1.20 g/L, respectively. Under the same culture conditions, the use of ultrasonic treatment resulted in a biomass yield approximately 3.34 times higher and an increase in intracellular and extracellular polysaccharide levels by 3.88 times and 4.25 times, respectively, compared to cultures without ultrasonic treatment. For the aerated submerged culture without

ultrasonic treatment, the biomass, intracellular polysaccharide, and extracellular polysaccharide yields were 18.16 g/L, 2.68 g/L, and 3.12 g/L, respectively, after 162 hours of culture.

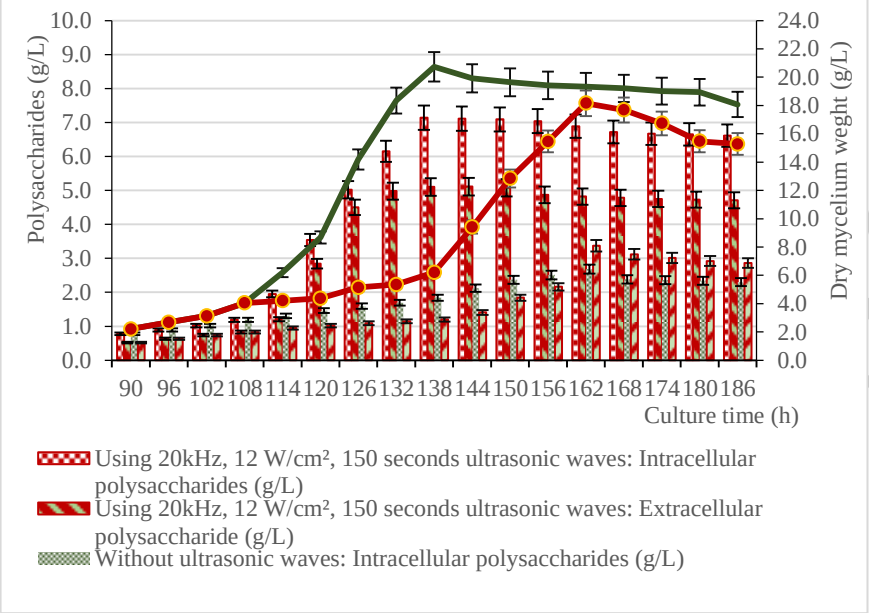

Figure 4. Effect of aerated Submerged Culture Duration of P.B91 on Biomass and Polysaccharide Production.

These results may stem from the positive biological effects of the ultrasonic pulsed wave field. In comparison with studies on submerged culture of *Phellinus linteus* [5, 9], These findings indicate that ultrasonic energy enhances biomass yield and shortens the culture duration required for P.B91 in submerged aerated culture, particularly for producing biomass, intracellular polysaccharides, and extracellular polysaccharides. The results highlight the potential of combining ultrasonic treatment with submerged culture technology for efficient production of P.B91 biomass and polysaccharides.

4. CONCLUSIONS

This study demonstrates the effectiveness of ultrasound energy in enhancing the productivity and reducing fermentation time for the P.B91 strain in the production of biomass, intracellular polysaccharides, and extracellular polysaccharides. The optimal conditions, involving ultrasonic waves at a frequency of 20 kHz, intensity of 12 W/cm², applied for 150 seconds after 108 hours of inoculation, resulted in a biomass yield of 20.73 g/L, with intracellular and extracellular polysaccharide levels reaching 7.14 g/L and 5.10 g/L, respectively. These results represent significant increases of 3.34 times, 3.88 times, and 4.25 times compared to the control (without ultrasonic treatment).

These findings underline the substantial improvements in both biomass production and polysaccharide yields achieved through ultrasonic treatment. From an industrial standpoint, this method offers several key advantages, including cost efficiency, time savings, and environmental sustainability. By reducing fermentation time, ultrasound treatment can lower energy consumption and operational costs. Moreover, the enhanced yields achieved with shorter

fermentation periods contribute to more sustainable production processes, reducing waste and optimizing resource use. In conclusion, the integration of ultrasound technology into submerged fermentation holds significant promise as a strategy for scaling up the efficient production of bioactive compounds, with potential applications in industries such as food, pharmaceuticals, and biotechnology.

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