Ultrasonic assisted fermentation for production of xylanase enzyme using locally isolated strain of *Bacilus badius*

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Ultrasonic assisted fermentation for production of xylanase enzyme using locally isolated strain of *Bacilus badius*

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Abstract. Ultrasonic assisted fermentation has been shown to improve the fermentation and product yield; however, less attention has been given to the xylanase production via ultrasonic assisted fermentation. The purpose of this study was to investigate the conditions in which ultrasonic has been employed to enhance fermentation reactions of interest to xylanase production. Previously, the xylanase production from locally isolated strain identified as *Bacillus Badius* was successfully carried out at an optimum condition. In this study, xylanase production was further explored by incorporated ultrasonic assisted fermentation. The study was conducted by varying the ultrasonic process parameters including treatment time, amplitude and duty cycle. Within the studied range, the highest xylanase production was 9.21 U/mL at duty cycle of 10 %, amplitude of 2 % and treatment time of 25 min. However, the production was 21 % lower than that the conventional optimized fermentation. This is due to unoptimized condition of the ultrasonic assisted fermentation as compared to the conventional one. Ultrasonication at the best process parameters is an effective and feasible way to enhance xylanase activity. In the future, we are looking forward to optimize the ultrasonic assisted fermentation to maximized xylanase production.

1. Introduction

Xylan can be found in the plant cell walls in hard woods plant which represents up to 20%–35% of the total dry weight of land plant [1]. Xylan is a heteropolysaccharide made up of a backbone of β-1,4-linked xylose which can be substituted to varying degrees with glucopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaryl side chain groups [2]. Due to its structural complexity, an array of hydrolytic enzymes is needed to break down these polymers to monomers. Several hydrolytic enzymes are required for the complete hydrolysis of xylan, in which the xylanases are most important. Xylanases are produced by diverse genera and species of microorganisms such as bacteria, fungi and actinomycetes [2].

Xylanase (endo-β-1, 4-xylanase) is defined as an extracellular enzyme. The main advantage of xylanase is its efficiency in the degradation of xylan into several xylose units by cleaving β-1,4-glycosidic bonds of xylan backbone in a random manner. The foremost industrial application of xylanase is involved in chlorine-free bleaching process in pulp and paper industry whereby xylanase is added into the pulp to degrade xylan found within the lignin residuals [3].

Xylanase production has been studied extensively in the laboratory scale. All of these studies were focused on the conventional fermentation. For example, Gautério et al. [4] using *Aureobasidium pullulan* while Nkohla et al. [5] used *Bacillus cereus* to produce xylanase in a submerged fermentation. On the other hand, Kumar et al. [6] using *Aspergillus niger* in solid state fermentation for their study in xylanase production. However, xylanase production via ultrasonic assisted fermentation has never been reported. Ultrasound has been found to be an efficient tool to improve the fermentation process. This is because ultrasound assisted fermentation has long studied by various researchers in various fields. Altogether were reported with positive effects. Bochu et al. [7], proved the influence of low intensity ultrasonic waves on the total accumulation of Ca^{2+} in *Saccharomyces cerevisiae* cells. In addition, ultrasound enhance the effusion and accumulation of microbial metabolism products leading to higher maximum yield. Nitayavardhana et al. [8] studied the effects of ultrasound on ethanol yields from chips of cassava, a crop in tropical or subtropical regions. They found that the fermentation time could be decreased by nearly 24 hours for sonicated samples to achieve the same ethanol yield as non-sonicated samples. Sonication not only reduced the fermentation time but also amplify ethanol production.

In this study, we explored the conditions in which ultrasonic has been employed to enhance fermentation reactions of interest to the xylanase production. The use of ultrasonic towards the small-scale xylanase production under submerged fermentation, particularly by the use of ultrasonic generator with treatment time (5 to 25 min), amplitude (1 to 5 %) and duty cycle (10 to 50 %), were discussed.

2. Materials and methods

2.1. Working stock

Bacillus badius was picked and streaked on agar slant by using inoculating loop and incubated at 37 ℃ for 24 h [9]. Then, agar slants were labelled and kept at 4 ℃ as working culture.

2.2. Broth media

Broth medium was prepared by dissolving 4 g of glucose, 4 g of peptone, 0.8 g of dipotassium phosphate, 0.12 g of magnesium phosphate and 1 g of ammonium sulphate in 400 mL of Sorensen's phosphate buffer [10]. Solution was stirred with magnetic stirrer to ensure homogeneity and autoclaved at 121 ℃ for 15 min. Sterile solution was then labelled and kept at 4 ℃ until further use.

2.3. Fermentation media

Broth medium was prepared by dissolving 20 g of xylan, 20 g of peptone, 4 g of dipotassium phosphate, 0.6 g of magnesium phosphate and 5 g of ammonium sulphate in 2000 mL of Sorensen's phosphate buffer [11]. Solution was stirred with magnetic stirrer to ensure homogeneity and autoclaved at 121 ℃ for 15 min. Sterile solution was then labelled and kept at 4 ℃ until further use.

2.4. Inoculum development

A loop of *B. badius* was inoculated from working stock into 100 mL broth medium and incubated for 8-10 h at 150 rpm and 37 ℃ [10]. After that, optical density (OD) of the inoculum was checked by using UV-vis spectrophotometer at 600 nm. The inoculum that have OD 2.0 was used as the vegetative cells for inoculum sources.

2.5. Conventional fermentation

Xylanase fermentation was carried out by submerged fermentation in 250 mL conical flask with 50 mL working volume (47.5 mL of fermentation media was mixed with 2.5 mL inoculum). The fermentation was carried out using the optimized conditions previously studied by Rosli [12] as shown in Table 1.

Table 1. Optimized fermentation condition.

Culture condition	Xylanase production condition
Initial pH of media	7
Temperature (℃)	38
Agitation speed (rpm)	120
Incubation period (h)	30

2.6. Ultrasonic assisted fermentation

Ultrasonication was commenced 12 h after inoculation of conventional fermentation. Sonication treatment was carried out with three variable manipulations at five different levels, which were treatment time, amplitude and duty cycle [7]. The level of each variable is shown in Table 2. The experiment was conducted for each variable using one factor at a time (OFAT) method.

Table 2. Manipulated variables with five levels for ultrasonic assisted fermentation.

Run	Variable	Five Different Levels					Fixed Variable
1-5	Treatment time (min)	5	10	15	20	25	Amplitude: 2% Duty Cycle: 10%
6-10	Amplitude (%)	1	2	3	4	5	Treatment time: 25 min, best from run 1-5 Duty Cycle: 10%
11-15	Duty cycle (%)	10	20	30	40	50	Treatment time: 25 min, best from run 1-5 Amplitude: 4%, best from run 6-10

2.7. Xylanase harvesting

The crude enzyme was harvested by centrifugation at 10,000 rpm for 15 min at 4 ℃. Then, the clear supernatant was collected and stored as crude enzyme at −20 ℃.

2.8. Xylose Standard Curve

2 mg/mL xylose working stock were prepared by mixing 0.4 g xylose in 200 mL phosphate buffer. Xylose working stock was then diluted into different xylose concentration of 1 mg/mL, 0.8 mg/mL, 0.6 mg/mL, 0.4 mg/mL, 0.2 mg/mL and 0.0 mg/mL. 0.5 mL of each concentration was pipetted to 1 mL of 0.05 M phosphate buffer in a test tube and incubated at 50 ℃ for 10 min After incubation period, 3 mL of DNS solution was added and incubated for 5 min at 100 ℃. Then, the sample was cooled down by placing the test tube under running tap water and the absorbance of the sample was read using UV-Vis spectrophotometer at 520 nm wavelength. The standard curve of absorbance vs. xylose concentration was then plotted [13].

2.9. Xylanase assay

0.2 mL of crude enzyme was added in a test tube. 0.5 mL of 1 % xylan solution and 0.3 mL of 0.05 M phosphate buffer were added to the test tube and then mixed well. The test tube was then incubated for 10 min at 50 ℃. After incubation period, 3 mL of DNS solution was added and incubated for 5 min at 100 ℃. The sample was cooled down by placing the test tube under running tap water and the absorbance of the sample was read at 520 nm. Enzyme blank (0.8 mL of 0.05 M phosphate buffer added to 0.2 mL crude enzyme) was prepared and treated the same as the crude enzyme tube [13]. The enzyme activity was calculated by using Equation (1).

$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{mL}}\right)=\left(\text{final absorbance -}\ \frac{\text{y-intercept from xylose standard curve}}{\text{slope from xylose standard curve}}\right)\times \frac{\text{dilution factor}}{\text{sample volume}}\times\frac{1}{\text{reaction time}}\times\frac{1000\ \mu\text{g}}{1\ \text{mg}}\times\frac{1\ \mu\text{mole}}{180.16\ \mu\text{g}} \quad (1)$$

2.10. Statistical analysis

All experiments were performed in duplicate and the average values are reported. The average and standard deviation values were calculated using the respective functions (AVERAGE, STDEV) available in Microsoft Excel and the maximum difference among all values was less than 5% of the mean. Statistical significances from the control experiment was evaluated using t-test comparisons at a level of 0.05 ($P < 0.05$).

3. Results and discussion

3.1. Influence of ultrasonic treatment time on xylanase activity

Figure 1 shows the xylanase activity with various ultrasonication time. Ultrasonication time has extensive impact on xylanase production. Increment in ultrasonication time could be due to an increase in shear forces applied on the *B. badius* which causes more deformation. This resulted in higher diffusion of nutrients across cell membrane. It is expected to have high xylanase activity with increasing sonication time.

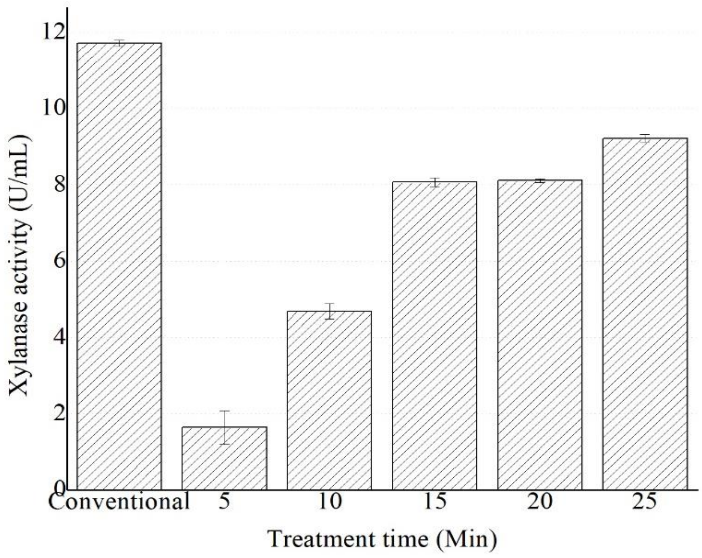

Figure 1. Xylanase activity with different ultrasonication time. Error bars represent standard errors; the results from the two experimental runs were significantly different ($p < 0.05$).

From the figure, xylanase activity increases drastically as ultrasonication time increase from 5 to 15 min, about 0.573 U/mL for each time increased. After that, no significant raised at high sonication time. These results could be explained as an increase in sonication treatment time led to increase of cell disruption. It has been attributed to the formation of pores and holes in the microbial cell membrane, thus improving their permeability in a process known as sonoporation [14]. Although these pores lead to sub lethal injury to the microbial cell, they provide a channel for transport of nutrients across these membranes [15]. Treated cells with minor physical damage can recover from this injury and subsequently increase in number during the fermentation.

Based on Figure 1, xylanase activity of conventional fermentation was the highest than that the ultrasonic assisted fermentation. This is because the ultrasonic condition not the optimal for ultrasonic assisted fermentation. On the other hand, conventional fermentation follows the optimal fermentation conditions. Therefore, the microbes in the conventional fermentation were very active than the ultrasonic assisted fermentation which causes the production of xylanase in conventional fermentation higher than the latter. Irfan, et al. [10], studied optimization of process parameters for xylanase production by *Bacillus* sp. in submerged fermentation and reported cultural properties played a pivotal role in enzyme production. Eventually, it causes high xylanase activity in conventional fermentation.

3.2. Influence of ultrasonic amplitude on xylanase activity

The effect of ultrasonic amplitude on xylanase activity is shown in Figure 2. From the result, xylanase activity was slightly improved as ultrasonic amplitude increased from 1 to 3 %. The xylanase activity increases drastically as amplitude increased from 3 to 4 % with 8.64 U/mL.

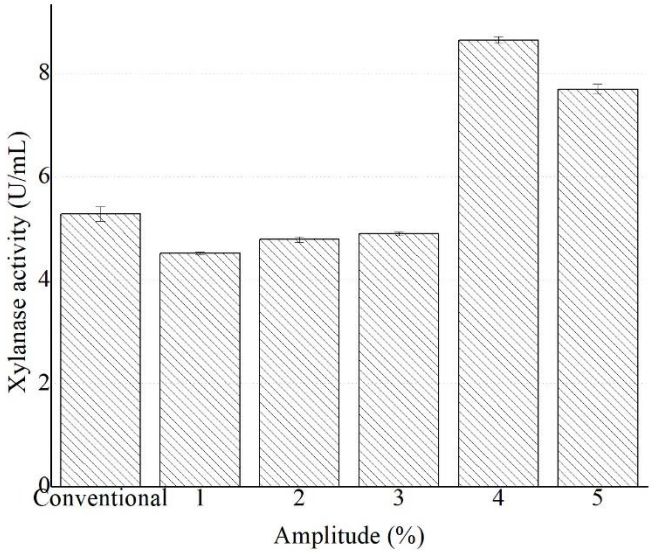

Figure 2. Xylanase activity with different ultrasonic amplitude. Error bars represent standard errors; the results from the two experimental runs were significantly different ($p < 0.05$).

These results could be explained as the increase of ultrasonic amplitude lead to an increase in shear force applied on *B. badius*'s cells. It has been attributed to loose cell bunches formed in the process of microbial culture. It enhances nutrient utilization by *B. badius*'s cells resulting in high of cell biomass and leading to higher productivity of xylanase. Besides, increase in ultrasonic amplitude lead to an increase of foam stimulated by ultrasonic in fermentation media. As the quantity of foam increases, the exchange area between gas and liquid in fermentation media also increases. Sufficient exchanges between gas and liquid leads to improved oxygen transfer rate. It is beneficial to the growth of *B. badius* and improve the production of xylanase. When the amplitude was increased from 4 to 5%, the xylanase activity starts to decrease. As *B. badius*'s cells sonicated at high amplitude, the treatment provoked puncturing of cell membrane with leakage of high intracellular content as well as damage at the subcellular level [16]. Eventually, kill the microbes and affect the production of xylanase.

Sonication did increase the fermentation media temperature. This is because ultrasound is an alternation of high and low frequency waves which may cause cavitation. During the low frequency phase, these bubbles grow, whereas in high frequency phase they are compressed and therefore implode. Imploding causes a temperature rise and heat exchange within fermentation media [15]. As the temperature increases, fermentation rate accelerates. With increased in fermentation rate, more xylanases are produced. The intensity of sonication is directly proportional to the ultrasonic amplitude.

Thus, an increase in amplitude of ultrasonic will lead to an increase in the sonochemical effects. The sonochemical effects are known as formation of microcavities, development of microcavities and implosion of microcavities. When fermentation media are exposed to the ultrasonic irradiation, bubbles in the media could be imploded by acoustic fields. Cavitation's bubble collapse can induce a shock wave in the media and drive the rapid impact of the liquid to the surface of the microbes and substrates [15].

Higher amplitudes are not always needed to obtain the desired effects especially in the case of microbial reactions. In addition, high amplitudes of sonication can lead to worsening of the ultrasonic transducer, which results in too much cavitation and poor transmission of the ultrasound through the liquid media. However, in this case, the amplitude should be increased for the fermentation media. Fermentation media frequently contain carbon sources that may render the medium non-Newtonian and relatively viscous. So, as the viscosity increases, the resistance of the liquid medium for the movement increases. As a result, a high intensity is generally required to set the ultrasonic device to obtain the necessary mechanical vibration in order to promote cavitation.

3.3. Influence of ultrasonic duty cycle on xylanase activity

The effect of ultrasonic duty cycle on xylanase activity is shown in Figure 3. Duty cycle is the fraction of one period in which ultrasonic system is active. A period is the time takes for an ultrasonic system to complete an on-and-off cycle. Duty cycle is commonly expressed as a percentage or ratio. A duty cycle of 10 % was equivalent to sonication for one second followed by rest period (no sonication) of 9 s. A sonication duty cycle of 100 % meant uninterrupted sonication.

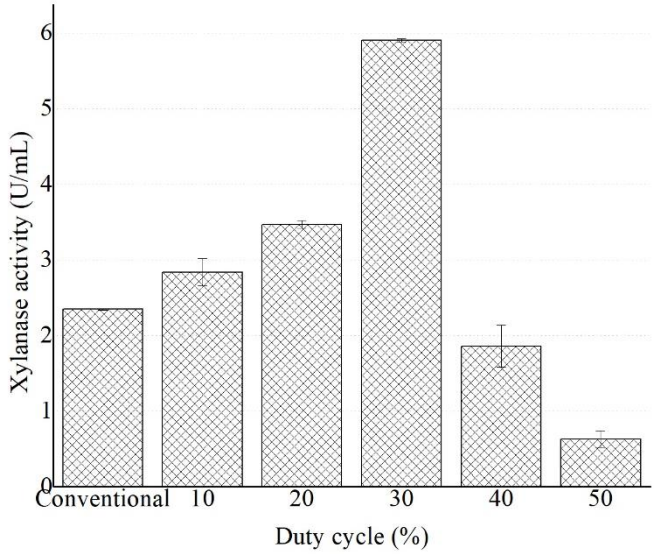

Figure 3. Xylanase activity with different ultrasonic duty cycle. Error bars represent standard errors; the results from the two experimental runs were significantly different ($p < 0.05$).

Figure 3 shows xylanase activity was improved when the duty cycle increases from 10 to 30 % with 5.91 U/mL. As the fermentation media sonicated at high duty cycle, physical or mechanical effects of ultrasonic at this range are capable of altering substrates properties (e.g., disrupting the physical integrity of substrate) through generation of immense shear gradient in the media and causes softening of the substrates [8]. So, it helps *B. badius* takes up the substrates (carbon source), improve the extraction of xylanase in fermentation media. However, the xylanase activity decreases drastically as duty cycle go beyond 30%. These results could be explained as the duty cycle increases; the cumulative average ultrasound dose could be increased. Eventually, the temperature of fermentation media increases. As the temperature increases, vapour pressure of the solvent also increases and therefore,

more solvent vapour fills the cavitation bubbles, making the collapse less violent, and reducing sonication effect [16].

Based on Figure 3, 30 % duty cycle resulted in an improvement in the xylanase activity by 60 % than that the conventional one. Higher duty cycle resulted in negative result as the temperature of fermentation media increases while the fermentation media sonicated at high duty cycle. Higher temperature leads to the deactivation of the microbes. Although temperature plays an important role in increasing the rate of reaction, it can also lead to the decrease in the microbial activity when increased beyond a certain optimum value.

Velmurugan and Incharoensakdi [17] studied the effect of ultrasound on enzyme production, enzymatic hydrolysis and simultaneous saccharification and fermentation of sugarcane bagasse for ethanol production. They reported an improvement in xylanase activity between fermentation with ultrasonic than that without, as much as 14%. This lead to 33% of increased in ethanol yield. Li et al. [18] applied an ultrasonic treatment to solid state fermentation of multienzyme production from *A. japonicus*. They recorded a 30% increase in xylanase production when the treatment was applied. These findings supported the current study outcome where an increase of more than 100% was observed in xylanase activities when ultrasonication was applied to the fermentation.

4. Conclusion

The effect of ultrasonic process parameters on the production of xylanase with ultrasonic assisted fermentation using *B. badius* was determined. There were three ultrasonic process parameters that have been investigated. The parameters studied were treatment time, amplitude and duty cycle. The best treatment time within studied range on the xylanase production was 25 min with 9.21 U/mL of xylanase activity. It was also observed that the xylanase production was the best at 4% amplitude with 8.64 U/mL of xylanase activity. For the effect of duty cycle on the xylanase production, the best ultrasonic duty cycle was found at 30% with 5.91 U/mL of xylanase activity. Further work can be continued to optimized the ultrasonic process parameters to further maximize xylanase production.

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