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Optimization of growth temperature for the production of cellulase in Myceliophthora thermophila

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OPTIMIZATION OF GROWTH TEMPERATURE FOR THE PRODUCTION OF CELLULASE IN MYCELIOPHTHORA THERMOPHILA

by

Daning Lu

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Chemistry

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Shuvendu Das and Mark A. Arnold
Thesis Mentor

Spring 2018

All requirements for graduation with Honors in the
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Claudio J. Margulis
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Abstract

Myceliophthora thermophila can produce various cellulolytic enzymes that degrade the major polysaccharides of plant biomass. Cellulase (exoglucanase) is an enzyme produced by these cells and excreted in the supernatant of the culture broth during fermentation processes. Different temperatures were investigated as a means to increase production yield of the cellulase activity during seven days of fermentation. The highest yield was observed for the temperature range of 45-48 ˚C. The highest enzyme activity was 24.3 ± 0.2 U/mL of supernatant, which was observed during the culture growth at 45 ˚C. The enzyme activity decreased slightly at 48 ˚C to 21.5 ± 0.2 U/mL of supernatant. At 32 ˚C, 38 ˚C, and 41 ˚C, the enzyme activity decreased by nearly 50% (12.4-13.1 U/mL) in comparison to yields at 45 ˚C and 48 ˚C. For these measurements, one unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute at pH 5.0 and 50 ˚C. In addition, an uncommon buff color of culture and branched hyphal cells were observed in 45 ˚C and 48 ˚C fermentation flasks.

Key words: *Myceliophthora thermophila* (*M. thermophila),* fermentation, cellulase, exo-cellobiohydrolase, enzyme activity, temperature optimization, p-nitrophenyl β-D-cellobioside, p-nitrophenol, optical density (OD)

1. Introduction

Filamentous fungi are widely used for the production of plant biomass-degrading or hydrolyzing enzymes.[1] Efficient degradation of plant biomass requires the enzymes, such as cellulase, hemicellulose, and other polysaccharides-degrading enzymes, to produce fermentable sugars under ideal industrial conditions.[2-4] However, a complete degradation of polysaccharides of plant biomass to fermentable sugars requires long reaction times that can introduce possible contamination in the reaction mixture.[3-4] Increasing the fermentation temperature can accelerate reactions, but can also lower enzyme activity through denaturation pathways. Thermophilic fungi are resistance to thermal denaturation and are designed to operate at higher temperatures. Enzymes from some of the thermophilic fungi even can tolerate temperatures as high as 70-80 ˚C.[4] Thermophilic fungi show higher enzymatic activity than mesophilic fungi that require lower temperature to grow.[4-5]

The thermophilic fungus, *Myceliophthora thermophila* (*M. thermophila*), degrades wood and other cellulosic substances more efficiently than other thermophilic and mesophilic fungi.[6-9] Hence, *M. thermophila* has been chosen for this study. Optimal temperatures between 40 ˚C and 69 ˚C have been reported for production of various hydrolytic enzymes within *M. thermophila*.[1] In this study, the optimum temperature will be established for the production of cellulase enzymes from *M. thermophila*.

There are mainly three soluble extracellular cellulases, such as 1,4-β-endoglucanase, 1,4-β-exoglucanase, and β-glucosidase.[3-4] Endoglucanase works on the cleavage of β-1,4-sugar bonds, and exoglucanase is required to cut the non-reducing end of a cellulose chain and to release the β-1,4-glucosidase cellobiose.[1] Similar to exoglucanase, β-glucosidase cuts the glycosidic bonds between glucose and terminal non-reducing residues.[10] Exo-cellobiohydrolase

(1,4-β-exoglucanase) is produced in large amounts from *M. thermophila*. For this reason, an enzyme assay for exo-cellobiohydrolase is used as a marker for cellulase activity.

2. Materials and methods

2. 1. Materials and Instruments

M. thermophila strain was obtained from *Dyadic International Inc*. The cellulase activity assay requires p-nitrophenyl β-D-cellobioside, p-nitrophenol, sodium acetate, and sodium carbonate. All chemicals were purchased from *Sigma-Aldrich*. All culture medium components were also bought from *Sigma-Aldrich*. The 1.5 mL disposable UV-Vis cuvettes were used from Azzota Corp (10 mm of path length, 285-800 nm of wavelength range).

For sterilization, the autoclave *Hirayama HV-110* was used. *New Brunswick Scientific Excella E24 Incubator Shaker* was used for the first stage of fermentation. Second stage of fermentation was performed in a *New Brunswick Scientific I26 Incubator Shaker*. The microscopic images were taken under *AmScope Microscope.* The absorbance at 600 nm and 405 nm was measured by *Shimadzu UV-1280 UV-Vis spectrophotometer*. The concentration of glucose (in g/L) was measured by *GlucCell Glucose Monitoring System*. Samples were collected in *Eppendorf* 2 mL microcentrifuge tubes and centrifuged by a *BeckMan Coulter Microfuge 22R Centrifuge*.

2. 2. Fermentation medium

The medium was prepared from two solutions, solution A and solution B. Solution A was comprised of various inorganic nutrients: 6.30 g/L $(NH_4)_2SO_4$, 1.35 g/L $MgSO_4 \cdot 7H_2O$, 0.70 g/L KCl, 0.66 g/L $CaCl_2$, 13.80 g/L KH_2PO_4 monobasic, and 1.35 g/L trace elements. The trace

elements solution was prepared with 63.69 g/L EDTA disodium salt dihydrate, 22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 4.3 g/L $MnSO_4 \cdot H_2O$, 5 g/L $FeSO_4 \cdot 7H_2O$, 1.7 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, and1.5 g/L $Na_2MoO_4 \cdot 2H_2O$. The pH of solution A was adjusted to 7.0 with 6 M NaOH. Solution B contained 36.5 g/L D-(+)-glucose monohydrate in distilled water. Both solutions were separately sterilized by autoclaving for 30 minutes at a temperature of 121 ˚C. After sterilization, the solutions cooled to room temperature prior to mixing at the ratio of 3:1 (A: B, v/v). Then, 1 mL/L Biotin (6 mg/L), 2 mL/L 1000× Pen/Strep antibiotics (2 g of penicillin and 5 g of streptomycin in 100 mL distilled water), and 10 mL/L CAS (Casein Acid Hydrolysate Solution, 10% w/v) were added to the combined A & B solution.

2. 3. Shake flask fermentation

The first stage of fermentation was performed in two 2.8 L Erlenmeyer flasks separately. Each flask (400 mL of the medium) was inoculated with 2 mL glycerol seed culture of *M. thermophila*, which had been preserved at -80 ˚C. Both flasks were incubated at 35 ˚C and 225 rpm. After 60-72 hours, the incubation was ready when the glucose concentration was less than 5g/L and the PCV (Packed Cell Volume, % v/v) was greater than 10%. The PCV was measured after 10 mL of sample was centrifuged at 4 ˚C and 3,400× g for 20 minutes. In addition, culture from each 2.8 L flask was examined under the microscope to check the purity of the culture. The flask with pure, healthy and well-grown cells was chosen for the second stage of the fermentation.

For the second stage of the fermentation, 5 mL (10%, v/v) of culture from the chosen flask was inoculated to 50 mL of the medium in a set of 250 mL Erlenmeyer flasks. Six 250 mL flasks in total were prepared for each experiment where two different temperatures were studied.

Three flasks were incubated at one specific temperature and the other set of three flasks were incubated at the other test temperature. The shaking speed was maintained at 225 rpm for all flasks and temperatures. Cultures were grown at 32 ˚C, 38 ˚C, 41 ˚C, 45 ˚C, and 48 ˚C, respectively, for this study. The duration of each fermentation was approximately seven days.

2. 4. Sampling of fermentation cultures

Each day, 1.5-1.7 mL of culture broth was transferred from each flask to a 2 mL centrifuge tube. With appropriate dilution factor, the absorbance or OD (Optical Density) of the sample at 600 nm was recorded for each sample. The glucose concentration was also measured by using a glucose meter. In addition, microscopic images were taken under 40× magnification with 10% diluted samples. Afterward, the remaining amount of sample was centrifuged at 10,000× g and 22 ˚C for 5 minutes. The supernatant and pellet were stored at -20 ˚C for later use. The cellulase activity to be quantified was contained in the supernatant fraction.

2. 5. Assay Procedure for exo-cellobiohydrolase activity

The enzyme excreted in the supernatant from *M. thermophila* is a subclass of cellulase, called exo-cellobiohydrolase or exoglucanase. The substrate used for this assay was 8 mM p-nitrophenyl β-D-cellobioside in sodium acetate buffer (50 mM, pH5.0). The cellulase binds at the non-reducing end (ether group) and a D-glucose dimer (cellobiose) is cleaved, while a *p*-nitrophenol is released (Fig. 1). The standard curve of p-nitrophenol was prepared using 0, 5, 10, 15, 25, and 50 μM of p-nitrophenol (in triplicate) in 50 mM acetate buffer. 0.5 mL of 8 mM p-nitrophenyl β-D-cellobioside was mixed with 0.5 mL of p-nitrophenol standards and 10-fold diluted (with 50 mM acetate buffer) supernatant samples. Both standards and samples were

incubated in 2 mL micro-centrifuge tubes in a 50 ˚C water bath for 30 minutes, including the control that contained only 50 mM acetate buffer. At the end of incubation, 0.8 mL of ice-cold 0.6 M Na_2CO_3 was added to each tube to stop the reaction. After mixing briefly, all tubes were centrifuged at 10,000× g for 2 minutes. Absorbance of supernatant was then measured at 405 nm. The concentration of p-nitrophenol in the samples was determined from the p-nitrophenol standard curve. One unit (1 U) of cellulase activity is defined as the amount of cellulase releasing 1 μmol of p-nitrophenol per minute at pH 5.0 and 50 ˚C.

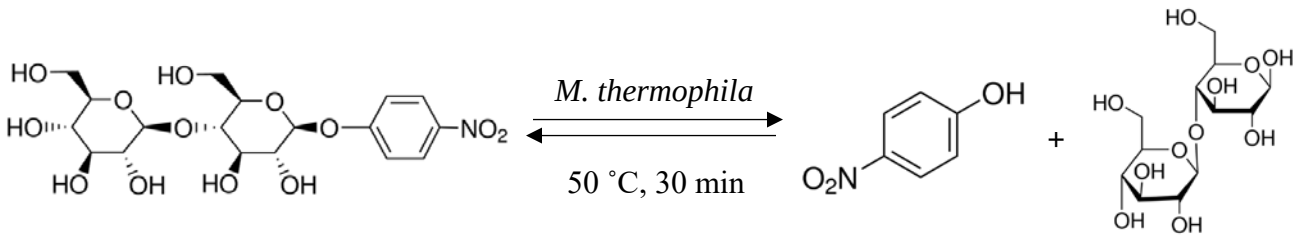

Fig. 1. Hydrolysis of p-nitrophenyl β-D-cellobioside produces p-nitrophenol and cellobiose by *M. thermophila*.

3. Results and discussion

3. 1. Effect of temperature on cellulase activity

For the first fermentation, the *M. thermophila* cells were expanded for approximately 145 hours in all experiments. The first pair of fermentations were performed in parallel at 32 ˚C and 45 ˚C, respectively. Higher cellulase activity was measured from the 45 ˚C fermentation compared to 32 ˚C fermentation (Fig. 2). At around 145 h (on the 7th day of fermentation), the cellulase activity is 26.0 ± 0.5 U/mL of supernatant for 45 ˚C and 12.4 ± 0.5 U/mL of supernatant for 32 ˚C. The estimated uncertainties of activity were calculated from the inverse

regression (shown in Appendix).[11] Results from the second set of fermentations reveals 21.5 ± 0.2 U/mL of cellulase activity when cells were grown at 48 ˚C compared to only 13.1 ± 0.2 U/mL at 38 °C. These first two experiments indicate that 45 ˚C produces the highest enzyme activity and that 48 °C also produces more enzyme compared to the lower temperatures tested.

A third experiment was designed to test temperatures between 41 ˚C and 45 ˚C to examine whether the optimal temperature is lower than 45 ˚C but higher than 38 ˚C. The higher cellulase activity again was observed at 45 ˚C (21.33 ± 0.09 U/mL), compared to 41 ˚C (12.85 ± 0.08 U/mL). In addition, after 96 h of fermentation, the rate of cellulase activity production began to decrease at 32 ˚C, 38 ˚C, and 41 ˚C, while it increased continuously during fermentations maintained at 45 ˚C and 48 ˚C.

Fermentations were repeated in duplicate at 45 ˚C to verify the production of cellulase at this temperature, and the results are presented with the previous two 45 ˚C fermentations (Fig. 3). The cellulase activity for the repeated sets at 45 ˚C was 24.9 ± 0.1 U/mL of supernatant. The quantitative variation for the repeated fermentations might be related to differences in the mechanical shaker. In all cases, the shaking speed was set at 225 rpm and the temperature was 45 ˚C, but mechanical deviations for different shakers could impact the growth of fungus and production of enzymes.

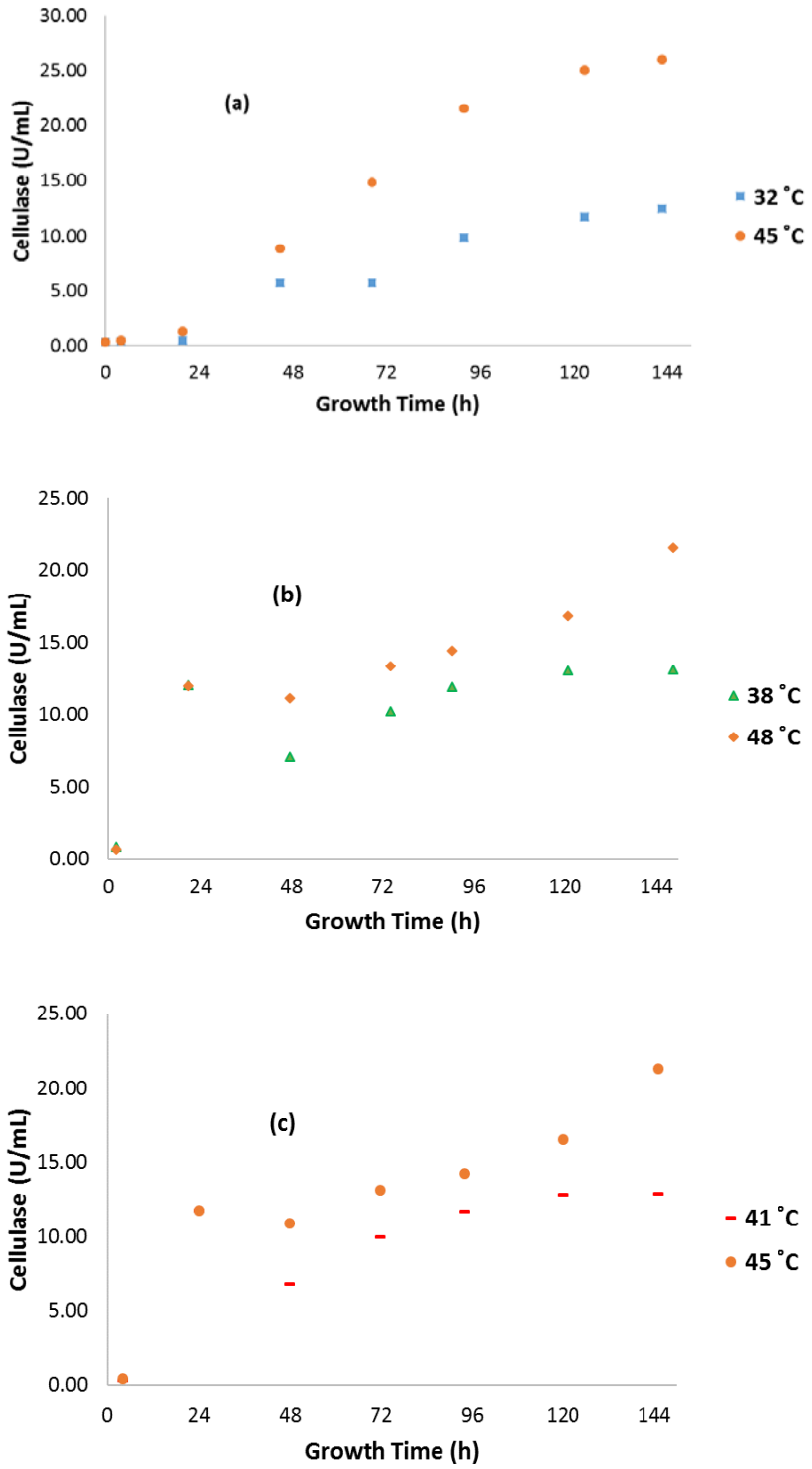

Fig. 2. Production of cellulase at different culture time. (a) Parallel fermentations of 32 ˚C and 45 ˚C; (b) Parallel fermentations of 38 ˚C and 48 ˚C; (c) Parallel fermentations of 41 ˚C and 45 ˚C.

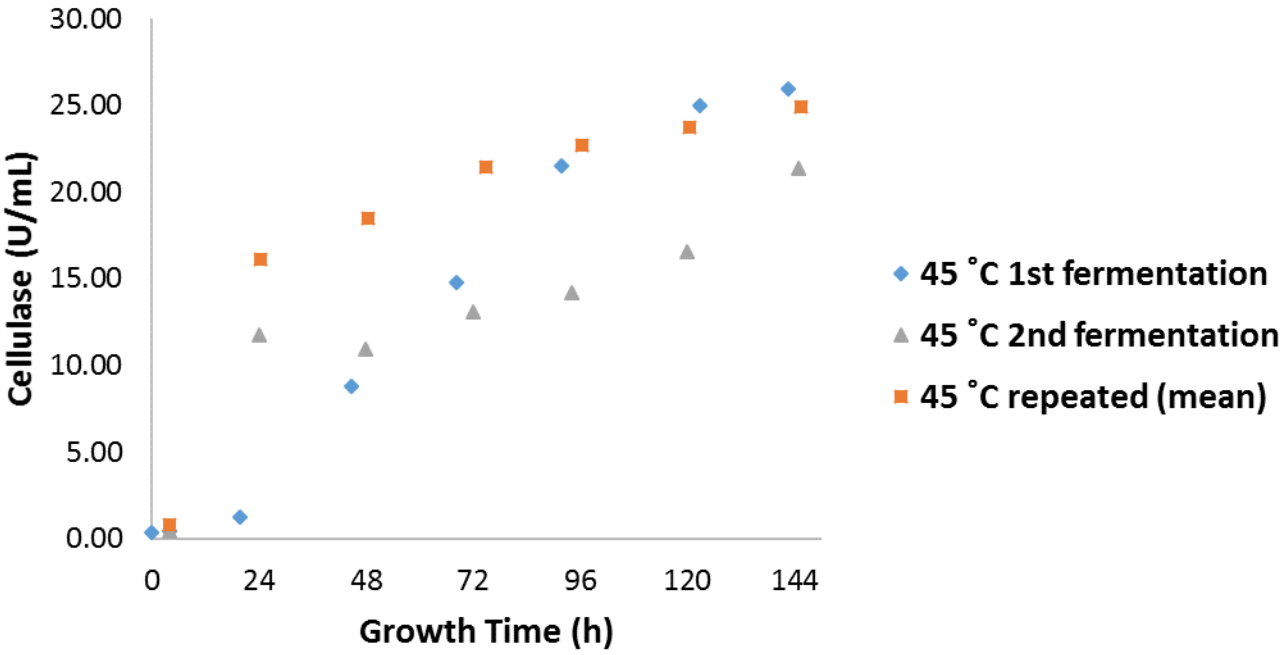

Fig. 3. All fermentations at 45 ˚C show an increasing trend of cellulase production

The comparison of final cellulase activities on the 7th day of fermentation indicates that 45 ˚C is the best temperature for the production of cellulase (24.3 ± 0.2 U/mL; Fig. 4). This activity value corresponds to the average of all fermentations at 45 ˚C. The error bar shows the propagated uncertainty. As shown in the histogram in Figure 4a, the cellulase activity does not improve significantly from 32 ˚C to 41 ˚C. However, from 41 ˚C to 45 ˚C, the cellulase activity increases by 1.89-fold. From 48 ˚C to 45 ˚C, the activity only improves by 1.13-fold but is still high (21.5 ± 0.2 U/mL) at 48 ˚C, relative to yields below 45 °C. The cellulase activity ratios shown in Figure 4b illustrates that the largest improvement is realized when going from 32 ˚- 41 ˚C to 45 ˚C, where the improvement is nearly a factor of two.

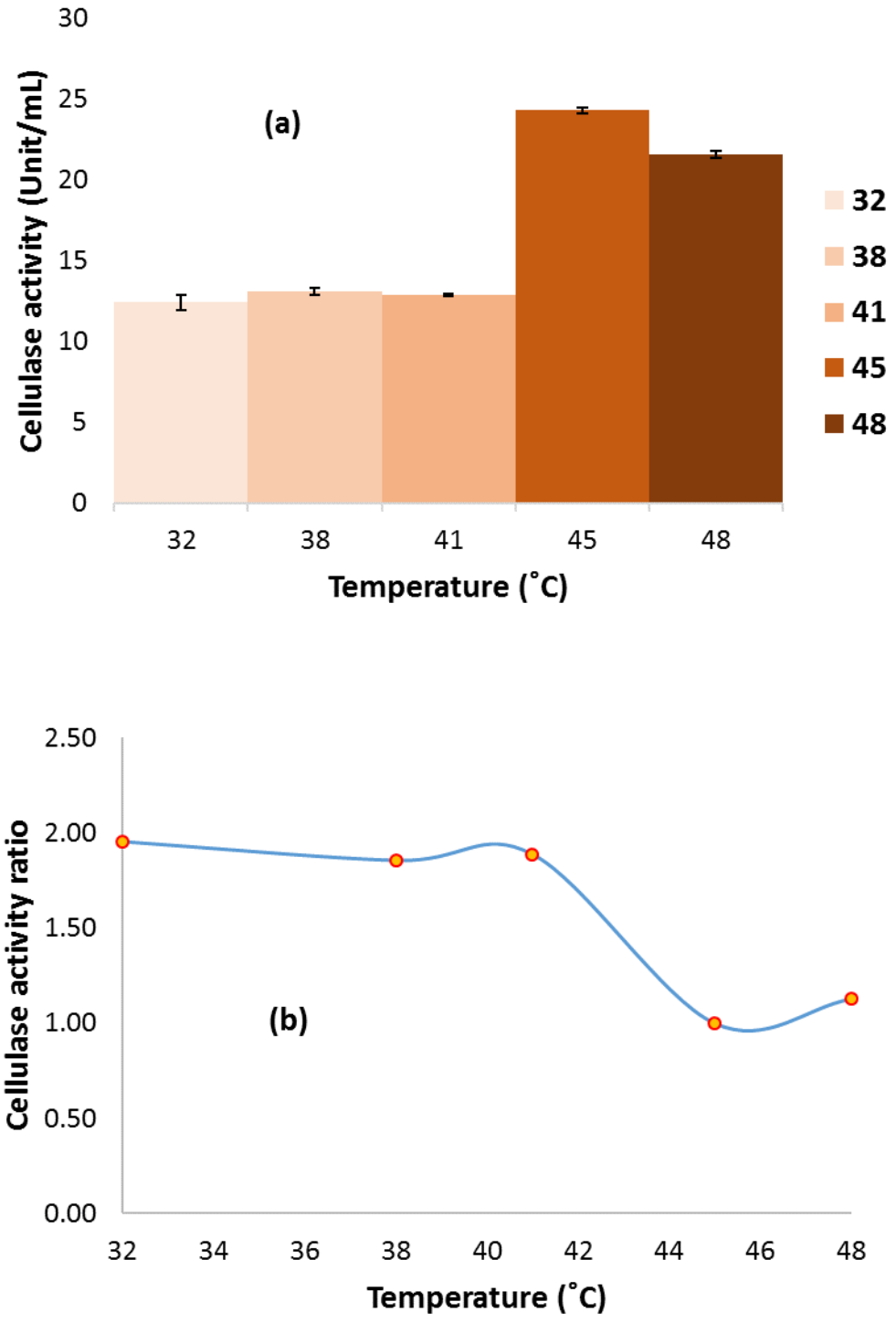

Fig. 4. (a) Comparison of cellulase activities on the final date (Day 7); (b) Cellulase activity ratio (activity at 45 ˚C : activity at other temperatures).

3. 2. Growth of M. thermophila and intriguing features

The growth curves of *M. thermophila* are based on the measurement of the OD (Optical Density) at 600 nm (Fig. 5). The lag phase was only observed at 32 ˚C from 2 to 40 h, and then the log phase was observed at 40-92 with the maximum OD of 8.23 at 92 h. After the log phase, the stationary phase was observed at 92-120 h. Finally, the death phase was observed when the rate of cell division was slower than the rate of cell deaths. At 38 ˚C and higher temperatures, the growth rate of *M. thermophila* was so high that the log phase was noticed immediately and no lag phase was observed.

The fastest growth in terms of maximum OD was observed at 45 ˚C (6.66, 23 h). The second fastest was 41 ˚C (8.61, 24 h), and then 38 ˚C and 48 ˚C were almost the same (7.64 and 7.17, respectively, at 74 h). Thus, the thermally stable *M. thermophila* can grow more at the lower temperatures (32 and 38 ˚C) but more slowly to reach the highest OD; nevertheless, it can grow much faster at the higher temperatures (45 and 48 ˚C) but less in OD maximum. At 41 ˚C, the OD reached the maximum only after 24 hours, which is also the highest value compared with other temperatures. Afterward, the OD decreased from 8.61 to 6.65 at 72 h and was remained in the stationary phase until the end of the fermentation.

Production of the cellulase enzymes continued during the entire fermentation (Fig. 4). After 48 hours of fermentation at 45 °C, the OD slowly decreases, while the cellulase activity slowly increases. The value of cellulase activity is not adversely affected by the gradual increasing rate of cell death. A possible reason for this observation is the breakage of dead cells releases the intracellular cellulase into the supernatant, thereby increasing the total amount of cellulase in the supernatant. Also, higher temperature might make the cell membrane more labile, resulting in a higher accumulation of cellulase in the supernatant.

Meanwhile, the initial glucose concentration in the fermentation medium was 9.125 g/L. After 4 hours of fermentation, the glucose concentration had dropped to 6.5-7.5 g/L at all tested temperatures. On the 2nd day, the level of glucose was lower than 0.3 g/L which is the detection limit of the glucose meter. This experiment was designed on the basis of batch fermentation without feeding glucose or other nutrients, so the fungus did not grow as well as it would have under fed-batch conditions. Optimal fermentation temperature (45 ˚C) is based on the shake flask batch fermentation without control of pH, [DO], agitation, and other parameters.

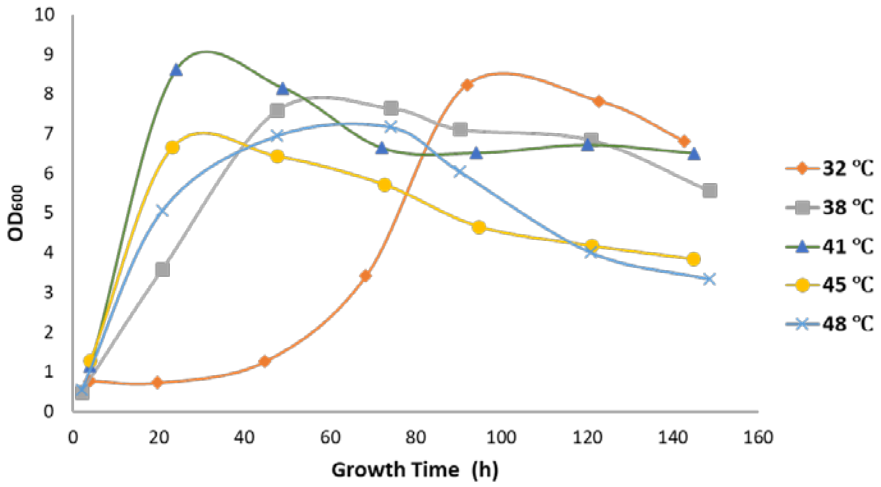

Fig. 5. Growth curves of *M. thermophila* at OD_{600}.

The first interesting observation during the *M. thermophila* fermentation pertains to the pigmentation of the culture. Initially, the color of the second stage culture was white. The color of the medium changed in the following manner with the different temperatures: at 32 ˚C and 38 ˚C, the medium displayed a cinnamon color; at 41 ˚C, the medium displayed a color between cinnamon and buff; at 45 ˚C and 48 ˚C, the medium displayed a buff color (Fig. 6). During conventional fermentations of *M. thermophila* at 38 ˚C, the color of culture broth turns into cinnamon and/or a darker color.

Mature colonies were reported to be cinnamon to light-brown color after three days of incubation at 45 ˚C.[12] Therefore, the buff color observed for the 45 ˚C fermentations was uncommon. The pigment of each culture could be related to the growth at different temperatures. As shown in the OD graph above (Fig. 5), at 32 ˚C, 38 ˚C, and even 41 ˚C, the stationary phase can still be roughly maintained during the late period of fermentation. The rate of cell division was approximately equal to the rate of cell death, and the live cells probably contained the pigment molecules showing the cinnamon color.

The normal cinnamon color of fungal cells disappeared during the 45 ˚C and 48 ˚C fermentations. This change in color might be due to the degradation of the pigments molecules at the higher temperatures. In addition, it took 48 hours for the 38 ˚C flasks to change color but 68 hours for the 32 ˚C flasks to change to the same color. As shown in the OD graph, live cells grow much faster at 38 ˚C and this temperature was acceptable for most of the cells to maintain structural integrity. The mixture of cinnamon and buff colors show that 41 ˚C was the temperature threshold of changing the pigment-dependent color of culture during the fermentation of *M. thermophila*.

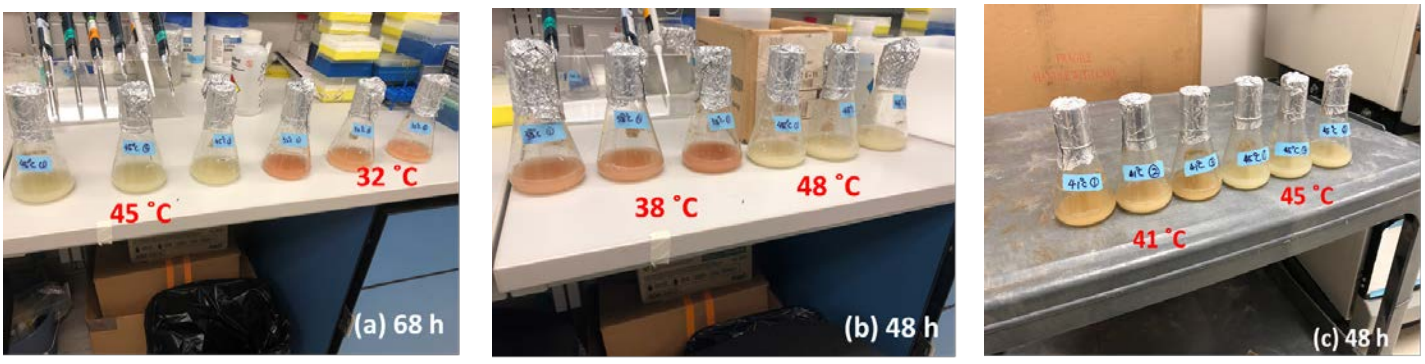

Fig. 6. Color of the cultures: (a) 45 ˚C buff, 32 ˚C cinnamon; (b) 48 ˚C buff, 38 ˚C cinnamon; (c) 45 ˚C, 41 ˚C between cinnamon and buff.

The second interesting observation corresponds to the morphology of the cells. In the regular fermentation process at 38 ˚C, the fungal cells are hyphal initially and gradually transform to unicellular shapes. In this study, microscopic images (40× magnification, 10% dilution of culture) showed the branched hyphal structure above a temperature of 45 ˚C throughout the entire fermentation process. At the same time, no contamination was observed at all investigated temperatures (Fig. 7 and 8). The microscopic images captured at 38 ˚C and 41 ˚C flasks clearly show the transformation of hyphal structure to mixture of hypha and unicellular shape of the cells after 91.95 hours and 72 hours of growth, respectively. From the growth curve (Fig. 5), we can conclude that this morphology change occurs when the cells enter the stationary phase. On the final day (Day 7) of fermentation, there were more unicellular cells at 38 ˚C and 41 ˚C, compared to the mixture of hyphal and unicellular cells, observed in the earlier time. In contrast, the cells grown at 45 ˚C and 48 ˚C show only the hyphal structure even at the death phase. At the end of fermentation, the hypha branch out from the main stem, rather than compress to be adherent to each other. The reason why the branched hypha exists over 45 ˚C is not clear. Organelles inside the cells can be observed in unicellular and short hyphal structures, but not in branched hyphal morphology which is transparent. Perhaps, branched hyphal structure of *M. thermophila* cells produces more extracellular enzymes in the medium, which resembles our observation on getting relatively higher cellulase activity at 45 ˚C and 48 ˚C.

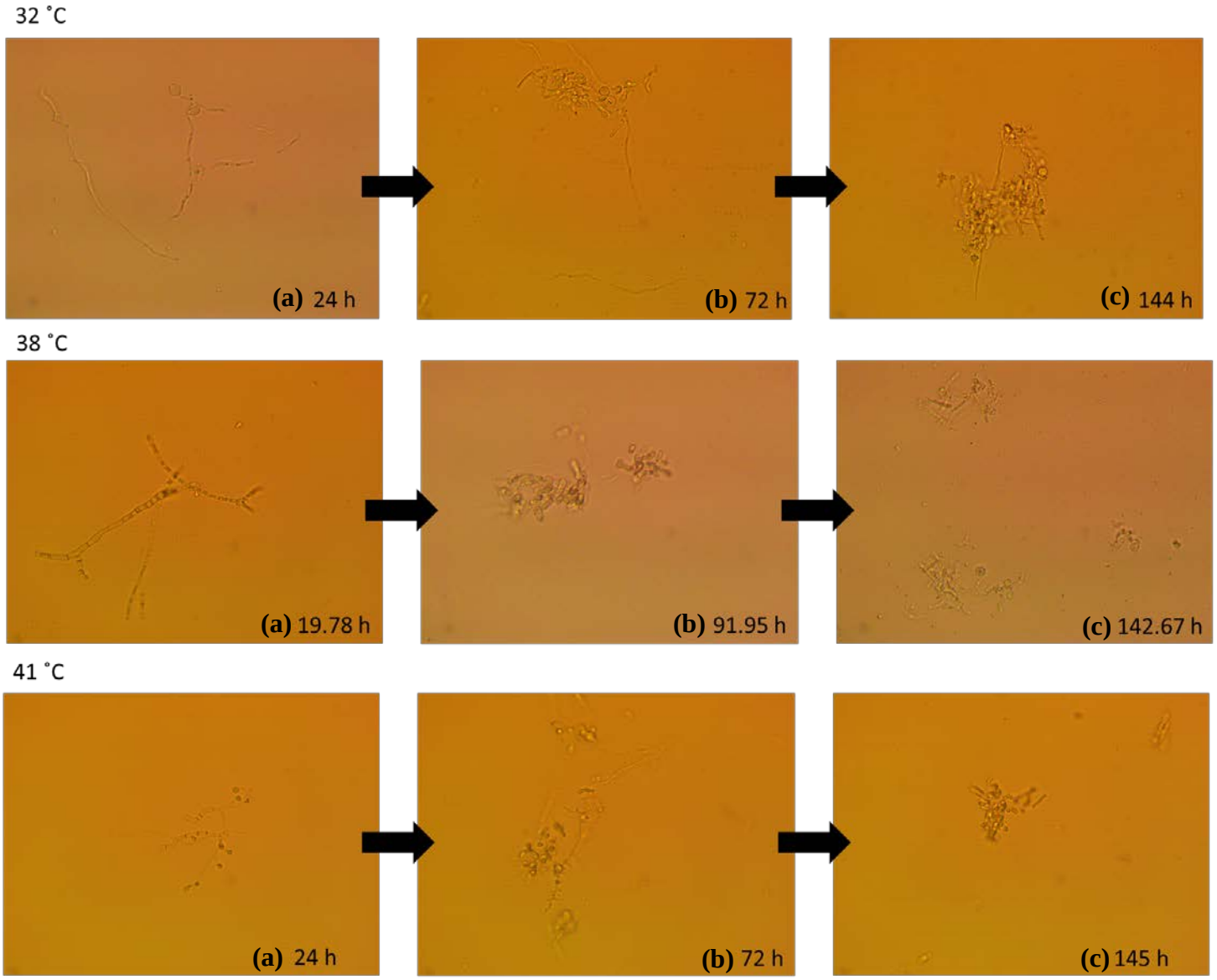

Fig. 7. Microscopic images (40× magnification, 10% dilution of culture) of 32 ˚C, 38 ˚C, and 41 ˚C grown culture: (a) hyphal cells mostly; (b) mixture of hyphal and unicellular cells; (c) unicellular cells mostly.

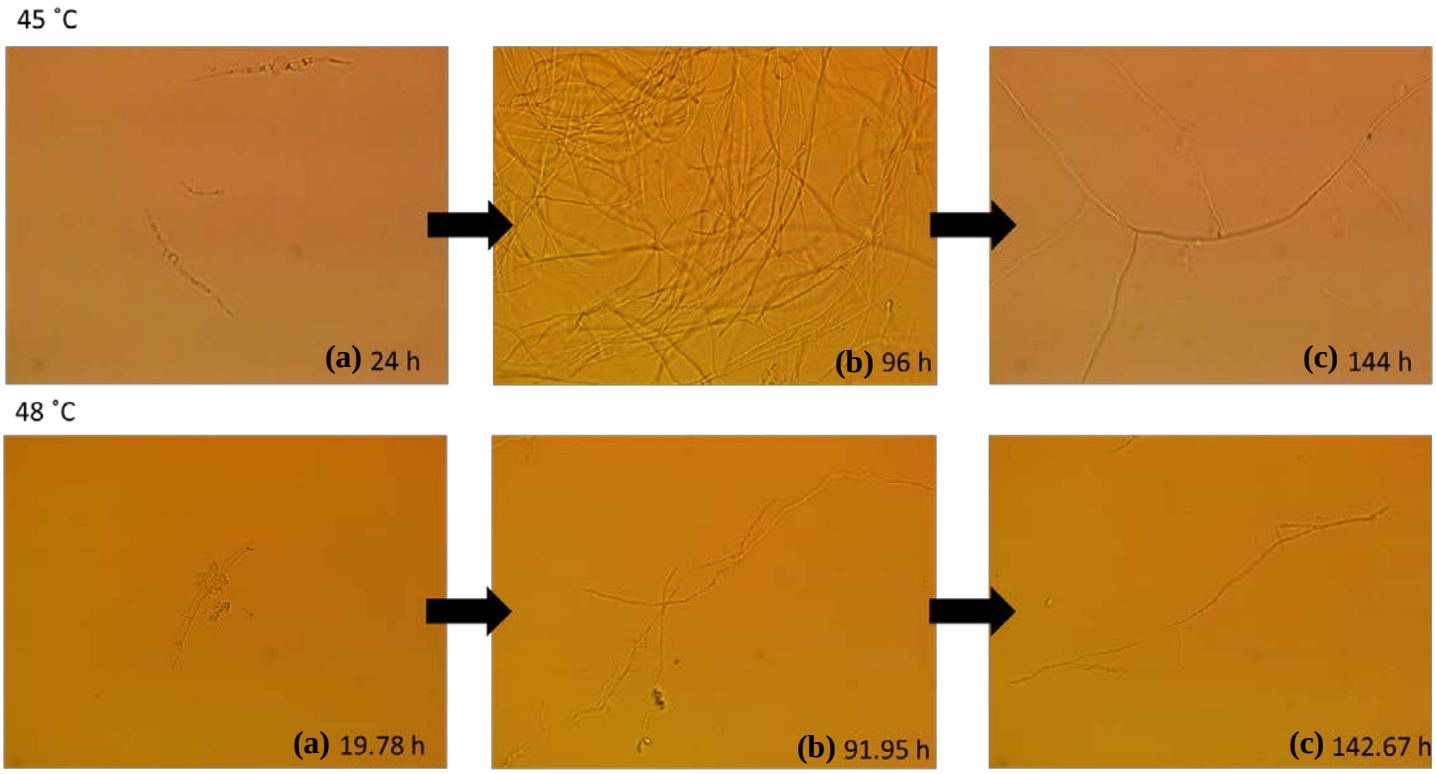

Fig. 8. Microscopic images (40× magnification, 10% dilution of culture) of 45 ˚C and 48 ˚C grown cultures: (a) hyphal cells mostly; (b) more branched hyphal cells; (c) branched hyphal cells mostly.

4. Conclusion

Highest cellulase activity (24.3 ± 0.2 U/mL of supernatant) is observed when *M. thermophila* is grown at 45 ˚C for seven days. At this stage, the culture medium is a buff color and cells show branched hyphal structure. The optimal temperature range for the production of cellulase in *M. thermophila* is 45-48 ˚C (21.5-24.3 U/mL). Cultures grown at 32-41 ˚C show a lower activity range (12.4-13.1 U/mL) than those measured for 45-48 ˚C fermentation temperatures. The higher production of cellulase at higher temperatures might be due to a change in the composition of lipid and phospholipid in the cell membrane, which leads to the transportation of more proteins across the cytoplasmic membrane to the supernatant.[13-14]

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Appendix: Sample calculation

Cellulase activity and estimated uncertainty from inverse regression.

The standard curve measurements for 1st 45 ˚C fermentation were shown below

Conc. of p-nitrophenol standards (μM)	Absorbance at 405 nm
0	0.002
5	0.087
10	0.163
15	0.238
25	0.422
50	0.875

The linear equation was

$$P = 0.0175 \times Abs - 0.009, R^2 = 0.9990,\ S_y = 0.01144 \qquad \textbf{Eqn. 1}$$

where P is the concentration of p-nitrophenol in μM, Abs is the absorbance at 405 nm, R^2 is the linear coefficient, and S_y is the estimated error of absorbance. At 142.67 h, the averaged Abs was 0.673 with the used volume of 50 μL (10X dilution factor), so the conc. of released p-nitrophenol was

$$P = 0.0175 \times 0.673 - 0.009 = 38.9\ \mu M$$

The supernatant was incubated in 50 ˚C water bath for 30 minutes, so the activity was

$$\frac{38.9}{30 \times 50 \times 10^{-3}}\ \mu mol/(min \cdot mL) = 26.0\ U/mL$$

Meanwhile, the propagated error of reverse regression is

$$S_x = \frac{S_y}{b_1} \times \sqrt{\frac{1}{k} + \frac{1}{n} + \frac{(y-\bar{y})^2}{b_1^2 \times \sum(x_i - \bar{x})^2}} \qquad \textbf{Eqn. 2}$$

where b_1 is the slope of linear equation, k is the number of standards, n is the number of replicate measurements, y is the measured absorbance, $\bar{y}$ is the absorbance average of standards, and $\sum(x_i - \bar{x})^2$ stands for the concentration deviation of standards. As a result, the propagated error for the calculated activity was

$$S_x = \frac{0.01144}{0.0175} \times \sqrt{\frac{1}{6} + \frac{1}{3} + \frac{(0.673-0.297)^2}{0.0175^2 \times 1637.5}} \times \frac{1}{30 \times 50 \times 10^{-3}} = 0.5\ U/mL$$