Effect of flow rate, duty cycle, amplitude, and treatment Time of ultrasonic regimens towards *Escherichia coli* harbouring lipase

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Abstract. A full factorial design (FFD) approach was conducted to assess the effect of four factors, namely flow rate, duty cycle, amplitude, and treatment time of ultrasonic regimens towards *Escherichia coli* harbouring lipase. The 22 experiments were performed as the following values with six replicates of centre point: flow rate (0.1, 0.2, and 0.3 L/min), duty cycle (0, 20, and 40%), amplitude (2, 6, and 10), and treatment time (10, 35, and 60 min). The FFD was employed as preliminary screening in shake flask cultivation to choose the significant factors (P< 0.05) for further optimisation process. In this study, zero duty cycle signified non-sonication of amplitude and no treatment time effect to the *E. coli* culture. Also, the designated flow rate and amplitude accordingly showed no effect towards the amount of dry cells weight (DCW). DCW was found significantly degraded after the exposure of high duty cycle and treatment time as other factors remained constant. Whereas for the lipase activity, no significant difference was observed in any main factors or interactions. Paired samples t-test confirms the result at a *p*-value of 0.625. This experimental study suggests the direct and continuous approach of sonication caused an adverse effect on the cells culture density.

1. Introduction

Lipases, or specifically triacylglycerol acylhydrolase (EC 3.1.1.3) can be found in all living organisms. Lipases act at the oil-water edge to transform water insoluble glycerides into smaller compounds which can be readily transported across the corresponding cell wall. Due to high potential of lipases in industry [1, 2], the increasing demands of lipases are shown by many productions from wild-type [3, 4] or genetically modified organisms [5-8]. In a comparison of wild-type and recombinant lipase production, the wild-type was found capable of consuming a broader range of oil-based substrate compared to the recombinant type [9]. It was presumed to the natural behaviour of wild-type in adaptation to its resources. While for the recombinant type, it produces higher activity but only for the desired protein translated in the recombinant gene. Hence, any production of lipase should consider the aim of its process condition. Since the production of recombinant protein in bioprocess have been
developed in the past 40 years [10], more strategies are needed to enhance the cultivation of the individual expression hosts. Among the current strategies are strain phenotype improvement [11], high throughput technology [12], and feeding strategy [13]. In 2003, Chisti also mentioned the feasibility of using ultrasound (US) in bioprocesses, including the recombinant protein recovery.

Despite the well-known use of sonicator for cell disruptions, degassing, cleaning, and homogenising, the use of ultrasound in enhancing cells cultivation is relatively new. Some of the recent US studies are focused on the effect of US treatments against enzymes [15-17], inactivation of enzymes and pathogens to avoid food spoilage [16], and assisting in enzymatic reaction towards insoluble substrate [18]. Owing to the potential of US in microbiology, some of the US parameters have been introduced in bioprocesses improvement from shake flask culture [19] to a laboratory bioreactor [20-22]. In this study, the use of US towards the small-scale cultivation of recombinant culture, particularly by the use of ultrasonic probes with medium flow rates, treatment time, amplitude, and the duty cycle, were discussed. To the best of our knowledge, this research was the first to study the effect of sonication towards a recombinant culture.

2. Materials and Methods

2.1 Microorganism

The recombinant E. coli harbouring lipase was obtained from the Laboratory of Biomolecular Medicine, Universiti Putra Malaysia. The lipase gene was gained from Staphylococcus hyicus and inserted into pTrcHis-TOPO vector by Hasan [23].

2.2 Growth medium and conditions

The recombinant E. coli was cultured in 500 mL Erlenmeyer flasks with working volume of 150 ml. Luria Bertani medium containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl was prepared and supplemented aseptically with 10 g/L glucose. The medium was adjusted to pH 7 prior autoclaving. In addition to keeping the plasmid carries the recombinant genes, sterilised filtered ampicillin was added until the final concentration of 100 µg/mL. Ten per cent of inoculum with OD600nm ~ 0.7 was added and the culture was shaken at 250 rpm under controlled temperature, 30°C. The culture was incubated for 3 h, later induced with 1 mM IPTG, and prolonged with an additional of 1 h incubation. After a total of 4 h incubation, the culture was transferred to a sterilised empty bottle with silicone bottle stopper and tubing. The tubing was connected to low volume continuous floccell (QSonica, USA) and the sonication was generated at 20 kHz. The culture was circulated using a peristaltic pump, Cole-Palmer Masterflex® L/S, USA. The schematic diagram of the experimental set is shown in figure 1. Samples were taken pre and post the treatment time (min) established by the experimental design and directly kept at 4°C for further analysis.

2.3 Full factorial design

A two-level full factorial design (FFD) was employed for the screening of important factors in enhancing the production of lipase from the recombinant E. coli. Flow rate (L/min), duty cycle (%), amplitude, and treatment time (min) at two different levels were selected as independent variables (table 1). The design generated 22 experiments, including six replicates of centre point, using Design-Expert software, version 7.1.6 (Stat Ease Inc., Minneapolis, MN USA). Two responses were included in the data, namely, DCW (mg/mL) and lipase activity (U/mL). The software is capable of building and analysing the experimental design by subjecting the data to the analysis of variance (ANOVA) for the determination of estimated effect and interaction and fitting the regression equation to the data as described previously in Saini et al. [24].
Table 1. Coded and actual values of the factors.

| Factor          | Low (-1) | High (+1) |
|-----------------|----------|-----------|
| Flow rate (mL/min), A | 0.1      | 0.3       |
| Duty cycle (%), B | 10       | 40        |
| Amplitude, C    | 2        | 10        |
| Treatment time (min), D | 10      | 60        |

\^ centre point is the average of minimum and maximum

Figure 1. Experimental setup combining a transducer ultrasonicator (A), a stand with a converter and a flocell (B), a peristaltic pump with sample tubing (C), and a bottle sample (D). Throughout the experiment, the bottle sample was kept in a biosafety cabinet.

2.4 Analysis

2.4.1 Dry cell weight
The dry cell weight (DCW) of E. coli culture was calculated from a standard curve of optical density (OD) versus the DCW. The OD was measured by UV-Vis 1800 Spectrophotometer (Hitachi, Japan) by comparing with fresh medium as blank. The standard curve was prepared by using triplicates of E. coli culture. Each replicate contained 15 mL of culture at OD ranging from 0.1 to 0.7. Each sample was then filtered using cellulose acetate membrane (47 mm diameter, 0.22 µm pore size) through a vacuum filtration set. Prior to filtration, the membrane was dried in an oven at 95°C until constant weight. After the filtration, the filter membrane with cells was carefully kept in the oven until constant weight. The dry cells weight was calculated by subtracting the initial filter membrane weight from the final weighing. By having the linear regression, the DCW was measured by the following Equation (1):

\[
DCW\left(\frac{mg}{mL}\right) = \frac{OD_{final}}{2.2341}
\]  

The standard equation in this study was found similar to the previous study by Koo et al. [25].
2.4.2 Lipase assay

Lipase activity was measured spectrophotometrically using p-nitrophenyl phosphate or pNPP (Sigma-Aldrich) as substrate. The assay was performed according to Kim et al. [26] with some modifications. The reaction mixture contained solution A (50 mM Tris-HCl buffer pH 8, 0.1% gum arabic, and 0.2% sodium deoxycholate) and solution B (10 mM pNPP in isopropanol). For the reaction, 100 µL of samples were added to 850 µL of solution A. The reaction was initiated by the addition of 50 µL of solution B and incubated at 35°C for 5 min. The reaction was stopped by the addition of 500 µL of 3 M of HCl. After centrifugation, 500 µL of clear supernatant was transferred to a cuvette and 1 mL of 2 M of NaOH was added. The mixture was read at 410 nm by UV-Vis 1800 Spectrophotometer (Hitachi, Japan) against a free enzyme mixture as blank. One lipase unit is defined as the amount of enzyme required to liberate 1 mmol of p-nitrophenol per minute.

3. Results

The recombinant cultures were cultivated in shake flasks and supplemented with ampicillin and IPTG for the expression of lipase. The factors chosen in this study were among the relevant variables discussed previously in other studies [16, 21]. ANOVA was carried out to determine the significant differences (p<0.05) among the factors. Throughout the experiment, the culture was initially cloudy, due to the growth of cells. After the sonication with amplitude and duty cycles, regardless the duration of engaged treatment time, the culture visually became apparent.

3.1 Effect of main factors and interactions for DCW_

Although the responses for DCW were measured as post-treatment (DCW, mg/mL) and percentage of change (DCW, %), both analyses have shown a similar pattern. Thus, for simplicity, only one response, specifically DCW, is chosen for further elaboration. Based on the ANOVA in Table 2, the model of DCW is found significant with two main factors, namely duty cycle and treatment time. Whereas, in two-factor interactions, a combination of duty cycle with amplitude and treatment time are significant. The rest of factors and interactions are not significant at p-value >0.05. The selected interaction studies between the four factors are shown in figure 2. According to the two top graphs in figure 2, the perpendicular lines clearly projects the interaction of the high level and low level of the amplitude and duty cycle. Duty cycle and treatment time demonstrate the same pattern. Hence, both interactions are significant while other factors kept at their constant values. Moreover, in the two bottom graphs in figure 2, the plateau corresponds to no significant different at any level. Parallel curves obtained for each factor indicates a lack of interaction in the experimental study [27]. However, from equation (2), the flow rate (A) shows a slight positive response. Thus, in the validation design, this factor was suggested at the highest level to obtain maximum DCW,

\[
DCW = +0.73 + 0.019 A - 0.46 B + 6.369 E^{-003} C - 0.073 D - 3.019 E^{-003} AB + 1.719 E^{-003} AC \\
+ 7.256 E^{-003} AD - 0.047 BC - 0.11 BD + 9.444 E^{-003} CD + 2.919 E^{-003} ABC - 0.023 ABD \\
+ 0.017 ACD + 0.016 BCD
\]  

(2)

3.2 Effect of main factors and interactions for lipase

From table 2, the ANOVA shows that the effect of factors at any term or interaction for the lipase activity are not significant. A negative predicted R² suggests that the overall mean is a better predictor of the response. Thus, a paired sample t-test was conducted to measure the pre-treatment and post-treatment time of sonication towards the lipase activity. The normality test by Shapiro-Wilk has passed at P=0.777 [28]. Using IBM SPSS for Window (Version 22, Armonk, NY.), there is no significant difference in the effect for pre-treatment (M=24.14, SD=2.59) and post-treatment,
(M=23.8, SD=1.53); \( t (21) =0.496, p=0.625 \). These results agree with the finding from the ANOVA and suggest that the treatment does not affect the lipase activity regardless the results shown by \( \text{DCW}_1 \).

3.3 Profiles of sonication outputs
Four sonication outputs are summarised in figure 3. The runs with no output were left blank thus only 14 runs out of 22 were observed on the sonication regimens. The intensity is calculated by using the average power in Watt divided by the area of replaceable membrane tip, where the sonication area is engaged with the sample [29]. The elapsed time was also measured by adding the “on” sonication process from the duty cycle setup. The duty cycle was set at 2 s “on” and 8 s “off” for a 20%, while 40% was set at 4 s “on” and 6 s “off”.

![Figure 2. Interaction studies between selected variables for response DCW$_1$.](image-url)
### Table 2. Three-way interaction ANOVA for DCW₁ and lipase production in recombinant *E. coli.*

| Source | DCW₁ | Lipase | Lipase |
|--------|------|--------|--------|
|        | Sum of squares | Mean square | F value | P-value | Sum of squares | Mean square | F value | P-value |
| Model  | 3.73 | 0.27   | 123.69 | $<0.0001^a$ | 37.64 | 2.69 | 1.38 | 0.3641 |
| A      | 5.92E-003 | 5.92E-003 | 2.74 | 0.1487 | 0.088 | 0.088 | 0.045 | 0.8385 |
| B      | 3.41 | 3.41   | 1579.33 | $<0.0001^a$ | 4.45 | 4.45 | 2.28 | 0.1821 |
| C      | 6.49E-004 | 6.49E-004 | 0.30 | 0.6031 | 0.080 | 0.080 | 0.041 | 0.8463 |
| D      | 0.08 | 0.08   | 39.18 | 0.0008$^a$ | 4.66 | 4.66 | 2.38 | 0.1735 |
| AB     | 1.46E-004 | 1.46E-004 | 0.068 | 0.8035 | 1.97 | 1.97 | 0.71 | 0.3543 |
| AC     | 4.73E-005 | 4.73E-005 | 0.022 | 0.8872 | 0.97 | 0.97 | 0.49 | 0.5083 |
| AD     | 8.43E-004 | 8.43E-004 | 0.39 | 0.5550 | 8.50 | 8.50 | 4.35 | 0.0820 |
| BC     | 0.04 | 0.04   | 16.37 | 0.0068$^a$ | 0.069 | 0.069 | 0.035 | 0.8572 |
| BD     | 0.18 | 0.18   | 84.37 | $<0.0001^a$ | 1.76 | 1.76 | 0.90 | 0.3791 |
| CD     | 1.43E-003 | 1.43E-003 | 0.66 | 0.4471 | 8.56 | 8.56 | 4.38 | 0.0813 |
| ABC    | 1.36E-004 | 1.36E-004 | 0.063 | 0.8099 | 1.51 | 1.51 | 0.78 | 0.4124 |
| ABD    | 8.66E-003 | 8.66E-003 | 4.02 | 0.0919 | 0.017 | 0.017 | 8.817E-003 | 9.282 |
| ACD    | 4.87E-003 | 4.87E-003 | 2.26 | 0.1837 | 3.28 | 3.28 | 1.68 | 0.2427 |
| BCD    | 3.95E-003 | 3.95E-003 | 1.83 | 0.2245 | 1.74 | 1.74 | 0.89 | 0.3820 |
| Curvature | 0.72 | 0.72 | 333.28 | $<0.0001^a$ | 9.109E-003 | 9.109E-003 | 4.663E-003 | 0.9478 |
| Residual | 0.01 | 2.16E-003 | | | 11.72 | 1.95 | 4.32E-003 | 4.32E-003 | 2.51 | 0.1743 | 6.14 | 6.14 | 5.51 | 0.0658 |
| R²     | 0.9961 | 0.7626 |
| Adjusted R² | 0.9871 | 0.2086 |
| Predicted R² | 0.4789 | -31.0285 |
| Adequate Precision | 31.693 | 6.215 |
| Coefficient of Variance % | 0.9961 | 5.87 |

A-Flow rate, B-duty cycle, C-amplitude, D-treatment time, $^a$-significant value of $p<0.05$
Figure 3. Sonication output profiles from experimental run, elapsed time ( ), total power ( ), average power ( ), and sonication intensity ( ).

The rapid shift of sonication increased the intensity and with longer treatment time, the cells were damaged [16]. Table 3 shows three responses with their respective predicted and experimental data. From the table, the Cook’s distance generated indicates the observations from the experimental are mostly higher than the limit. It was suggested by Altman and Krzywinski [30] that high leverage points may indicate the linear estimation must be restricted to a smaller region of the predictor region. In this study, only two main factors and two interactions are significant as described earlier in table 2. In addition to the screening factors, a Pareto chart is used to illustrate the importance of the factors complete with Bonferroni correction [31]. Therefore, figure 4 shows a Pareto chart of response coefficient for DCW and lipase activity. Figure 4a describes that the factors above Bonferroni level are significant according to the positive and adverse responses, while figure 4b shows that all the factors are below the t-value limit.

3.4 Validation of FFD
The software recommended the validation experimental design according to the factors range and responses target. Triplicates of recommended runs were conducted with a desirability of 0.8. The experiments gained 98.6% of the predicted values by the combination of flow rate at 0.3 L/min, duty cycle at 0%, amplitude of 10, and treatment time of 60 min. Hence, this combination suggests that the direct sonication was excluded in aiming high cell density. A maximum flow rate was proposed due to aeration and mixing of the aerobic culture in the bottle imitating the use of incubator shaker.

4. Discussion
This study shows different outcomes as compared to other studies due to a few reasons. The first reason is the approach of sonication with direct and indirect sonication regimens by using ultrasonicator probes compared to an ultrasonic bath [20, 22]. In this study, a floccell was attached to ultrasonicator probes, thus the sample was directly exposed to the sonication effect. While using an ultrasonic bath, the samples were contained in flask and reactor. Due to this, the glass wall of flask or
reactor might have hindered the acoustic effect. Thus, the effect was reduced compared to ultrasonicator probes. Furthermore, the probe sonication has localised effect compared to bath or tank type sonication [32]. The research also suggests that the use of ultrasonicator probe is more intense and efficient in the degradation process compared to an ultrasonic bath.

Another proposed reason is the sample volume. A small volume in the Erlenmeyer flask from this study compared to a larger volume in a bioreactor [20, 21] influenced the cultivation performance. However, previous research done by Avhad et al. [19] also used a small volume in a glass vessel for crude broth containing fibrinolytic enzymes. The different approach was the use of ultrasonic bath instead of ultrasonicator probe. The assisted ultrasonication in their study showed that the irradiation time of 5 min was the best to obtain high fold of fibrinolytic enzymes. The enzymes activity was started to deteriorate after 5 min due to excessive irradiation. This trend supports the current study for DCW\(_1\) which indicates that the irradiation does affect the cells and enzymes performances. In the following year, Avhad and Rathod [20] suggested that the treatment of sonication should be comparable to the volume size to minimise the decline of cells and enzymes.

**Table 3.** Observed and predicted responses in the experiments obtained by FFD.

| Run order | DCW\(_1\) (mg/mL) | Lipase (U/mL) |
|-----------|-------------------|---------------|
|           | Exp. | Pre. | Exp. | Pre. |
| 1         | 0.35 | 0.39 | 23.98 | 24.60 |
| 2         | 1.19 | 1.26 | 24.29 | 24.91 |
| 3         | 1.17 | 1.22 | 18.47 | 19.09 |
| 4         | 1.20 | 1.19 | 22.98 | 23.60 |
| 5         | 0.34 | 0.32 | 22.89 | 23.76 |
| 6         | 0.54 | 0.51 | 23.82 | 24.44 |
| 7         | 0.11 | 0.10 | 23.00 | 23.62 |
| 8         | 1.36 | 1.32 | 25.87 | 25.25 |
| 9         | 0.10 | 0.14 | 25.29 | 24.67 |
| 10        | 0.34 | 0.32 | 23.38 | 23.76 |
| 11        | 0.49 | 0.49 | 25.25 | 24.63 |
| 12        | 1.26 | 1.18 | 23.96 | 23.34 |
| 13        | 0.26 | 0.32 | 23.18 | 23.76 |
| 14        | 0.08 | 0.08 | 25.87 | 26.49 |
| 15        | 0.42 | 0.41 | 23.46 | 22.84 |
| 16        | 0.28 | 0.32 | 23.08 | 23.76 |
| 17        | 0.07 | 0.03 | 24.01 | 23.39 |
| 18        | 0.36 | 0.32 | 25.61 | 23.76 |
| 19        | 1.07 | 1.10 | 23.10 | 23.72 |
| 20        | 1.16 | 1.13 | 23.48 | 22.86 |
| 21        | 0.36 | 0.32 | 24.44 | 23.76 |
| 22        | 1.12 | 1.13 | 24.11 | 23.49 |

Exp. – Experimental, Pre. – Predicted, DCW\(_0\) – DCW at pre-treatment time, DCW\(_1\) – DCW at the post-treatment time.
Nevertheless, in this study, the lipase activity was found to be similar in all experimental runs. For this reason, the trend of lipase activity was not due to any of the manipulated variables. The pattern suggests that the cultivation was not fully expressing the lipase and the culture condition was not suitable for the lipase activity. It is assumed that the induction time for the lipase to express was not enough in this study. In common expression cultivation, it would take about 4 h after the induction to harvest the protein of interest. This condition might be due to the low pH throughout the fermentation duration (data not shown). As the experiments used Erlenmeyer flasks, the pH was set at 7 only before autoclaving, but was not controlled throughout the incubation period. As in conventional batch fermentation cultivation, the pH is reduced gradually from pH 7 to pH 5 because of the formation of by-products such as lactic acid and acetic acid. In other studies, by Wang et al. [33] and Gadge et al. [34], the optimum pH for lipase activity was suggested ideal between pH 6 and pH 8. Thus, the low pH cultivation in this study appears to cease the lipase activity and yield a similar pattern.

From previous discussion, the need for the controllable system includes the pH stability can be achieved using a bioreactor instead of Erlenmeyer flask. Also, the use of controlled conditions in the bioreactor aids survivability of culture against the sonication. Besides the above reasons, treatment time has also imparted an impact to the result as discussed earlier. Along with swift duty cycle regimen, the treatment time is suggested to be in short pauses as an alternative of continuous sonication. Dey and Rathod [35] and Dhanalakshmi and Nagarajan [32] recommended intermittent or pause sonication treatment rather than continuous to maintain the usability of the transducer and less power would be used.

5. Conclusions
The use of ultrasound in enhancing cell cultivations especially in harbouring recombinant protein was statistically designed and discussed in this paper. The outcome of the selected factors and level are shown to be significant to the post-treatment dry cell weight. From four factors, only duty cycles and treatment time affected the responses. While for the interactions, only two interactions, duty cycle-amplitude and duty cycle-treatment time were found to be significant. This study suggests that the use of ultrasonicator probe for the cell cultivation should be investigated in large volume with controlled parameters such as pH and dissolved oxygen for maintaining the cell growth. The ultrasonication approach also needs to be introduced carefully to avoid cell degradation and primary product
denaturation. Further data collection is also required to determine exactly how the ultrasound affects the lipase activity in recombinant *E. coli* cultivation.

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