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# KINETIC CORRELATIONS OF GAS-LIQUID MASS TRANSFER COEFFICIENT AND OXYGEN UPTAKE RATE OF HETEROLOGOUS PROTEIN CULTIVATION BY SONOBIOREACTOR (FRGS/1/2016/TK02/UMP/02/15)


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SUB-CLUSTER - CHEMICAL AND PROCESS ENGINEERING

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# 1.0 ABSTRACT

Sonobioreactor in this study is an integration system which includes the use of a sonicator with a bioreactor by the assembly of a peristaltic pump. The sonobioreactor was being applied to the production of a heterologous protein, lipase by the expression of a recombinant *E. coli*. The first objective of this study was to transform all the recombinant *E. coli* into BL21 host. The BL21 was found successful in carrying pTrcHis and pUC8, both carrying lipase gene from *Staphylococcus hyicus* and *Rhizopus oryzae*, respectively. Second objective is to investigate the characteristics of 2-L sonobioreactor for the use of recombinant *E. coli* cultivation. The sonobioreactor has shown some improvement of gas-liquid transfer (*kLa*) compared to the standalone system. Third objective is to determine the expression of lipase and plasmid stability of recombinant *E. coli* under sonicated and non-sonicated ultrasound regimens. The non-sonicated cultivation has shown higher lipase activity compared to the sonicated cultivation. The gradual sonication duty cycle has shown images of deformed cells of recombinant *E. coli* at 9K total magnification via SEM. Performances and kinetics of bioprocesses outcomes in this study may contribute to the current modelling of *E. coli* fermentations, especially for the recombinant protein production.

## 2.0 INTRODUCTION

Ultrasound (US) or ultrasonic waves are a sound wave that poses frequencies above 20 kHz. At these series, human could not capture the waves as it is above the audible range. The ultrasonic waves have high frequencies and short wavelengths. The use of ultrasound is expanding nowadays in medical, oil and gas industry, communication, agriculture, food, and transportations. In biotechnology especially fermentation technology, the practicality of ultrasound also offers great benefits along the processes and productions. Although the venture of ultrasound in this field is relatively new and currently exposed, some literatures shown that the use of ultrasound can be manipulate and further enhance the fermentation conventional methods to higher level. Using ultrasound, fermentation processes can accelerate the reaction speed, increasing in the reaction output, more efficient energy usage, switching of reaction pathway, performance improvement of phase transfer catalysts, and increase in the reactivity of reagents or catalysts. Most of the reports on the application of ultrasound in fermentation processes are in the area of improving the extraction, bio-reaction kinetics and enzymatic study [1-7]. Due to recent progress, the applications of ultrasound in microorganism and cells related production are recommended to further expand the potentials.

In current stream of molecular study, the cloning and genetic modification of fast-growing bacteria such as *Escherichia coli* (*E. coli*) is part of the recombinant protein harvesting. By using simple and well-researched host species like *E. coli*, the use of US can be deliberate in details for kinetics assessment. To the best of our knowledge, US is never been use in the production of any recombinant protein. Hence, this study proposed integration of US regimens in the fermentation process of recombinant strain, producing intracellular recombinant lipase. Like other physical irradiations, small change in the US intensity can damage and lyse cells [8] Consequently, the US regimens were expected to affect the stability of the recombinant plasmid and later the production of recombinant protein. Therefore, this study covers the effect of US from microscale level (DNA) up to the macroscale level (production in 2 L STB). Therefore, in this study, US is being carry out in 2 L stirred tank bioreactor (STB) for recombinant protein production.

# 3.0 RESEARCH METHODOLOGY

## *3.1 Preparation of Competent Cells*

Competent cells were prepared by calcium chloride ($CaCl_2$) treatment of *E. coli* BL21 (DE3) as described in Sambrook, Fritschi [9]. A single colony of BL21 from a lysogeny agar (LA) was inoculated into 5 mL lysogeny broth (LB). The LB was incubated at 37°C for 12 h at 200 rpm. A volume of 2 mL incubated culture was transferred into 50 mL centrifuge tube containing 20 mL fresh LB medium. The latter inoculated medium was incubated at 37°C, 200 rpm for 2 h. After the incubation, the centrifuge tube was centrifuged at 1000 x *g* for 5 min at 4°C. The pellet was resuspended in 10 mL of 75mM cold $CaCl_2$ followed by incubation on ice for 20 min. Then, the culture was centrifuged again at 4°C, 1000 x *g* for 5 min. After centrifugation, the supernatant was discarded and the pellet was resuspended in 2 mL of 75mM cold $CaCl_2$, followed by addition of 15% (v/v) glycerol. A volume of 100 µL of the competent cells was transferred into 2 mL cryovial and kept at -80°C for transformation process.

## *3.2 Plasmid Extraction of pTrcHis and pUC8.2-14*

One millilitre of overnight culture of *E. coli* Top 10 pTrcHis and *E. coli* ATCC® 68046™ pUC8.2-14 suspension were added into 1.5 mL microcentrifuge tubes. The microcentrifuge tubes were centrifuge at 10 000 x *g* for 1 min. The supernatant was discarded and only the pellets were used in the following procedure using *EasyPure*® Plasmid MiniPrep Kit, TransGen Biotech, China. The pellets were completely resuspended with 250 µL resuspension buffer which previously mixed with RNase A. Next, 250 µL of lysis buffer were mixed thoroughly by inverting the tubes 4-6 times. After 5 min, the procedure was repeated with 350 µL of neutralization buffer. The yellowish lysate was incubated for 2 min at room temperature. The microcentrifuge tubes were centrifuge at 10 000 x *g* for 5 min. The supernatant was carefully transferred into spin columns and then centrifuge at 10 000 x g for 2 min. The flow through were discarded and 650 µL of washing buffer were added into the spin columns. The spin columns were centrifuge again at 10 000 x *g* for 2 min and the flow through were discarded. The empty spin columns were centrifuge again at second time to completely remove residual of washing buffer. Next, the spin columns were placed into clean microcentrifuge tubes and

carefully 50 µL of elution buffer were added at the centre of column. The spin columns were incubated for 1 min at room temperature. Finally, the spin columns were centrifuge at 10 000 x *g* for 1 min and the isolated plasmid, pTrcHis and pUC8.2-14 were respectively stored at -20°C for future use.

### 3.3 *Plasmid Purity and Quantification*

Two microlitre of extracted plasmid was read against deionized water by NanoDrop Spectrophotometer, Thermo Scientific. The nucleic acids concentration was measured at 260nm while the purity was calculated using absorbance of 260/280 nm and 260/230 nm ratio.

### 3.4 *Transformation of Purified pTrcHis and pUC8.2-14*

*Escherichia coli* BL21 (DE3) competent cells (CC) were thaw on ice and immediately 2.5 µL of extracted plasmid was added into each 100 µL of CC. The mixtures were incubated for 20 min on ice. After incubation, a heat-shock treatment was performed by incubating the tubes at 42°C for 90 seconds [9]. After heat-shock, the tubes were incubated on ice for 1 min. A volume of 1 mL LB was added into the tube. The tube was incubated at 37°C, 200 rpm for about 1 h. After incubation, the tubes were centrifuged at 10 000x *g* for 1 min. and after centrifugation, 800 µL supernatant was discarded. The pellet was resuspended using the remaining supernatant. A volume of 100 µL of the transformant was spread onto LA plates containing ampicillin, 100mM and 50mM for transformant carrying pTrcHis and pUC8.2-14, respectively. The plates were incubated for 16 h at 37°C.

### 3.5 *Polymerase Chain Reaction and Gel Electrophoresis*

Polymerase Chain Reaction (PCR) was done respectively from colony culture of BL21 pTrcHis and BL21 pUC8.2-14 The PCR was also carried out using the extracted plasmids. A single colony of transformant BL21 pTrcHis was subjected for the colony PCR with primer forward of *Staphylococcus hyicus* lipase. Whereas a single colony of

transformant of BL21 pUC8.2-14 was using M13 forward pUC 40 and M13 reverse pUC 26. The single colony was diluted in 20 µL of sterile distilled water and mixed by vortex. PCR mixture reaction were added in 0.2 mL PCR tubes for a total of 20 µL with the respective volumes and final concentrations; Diluted colony, 1 µL (or extracted plasmid, 0.4 µL); Reverse primer, 0.2 µM; Forward primer, 0.2 µM; and *2xEasy Taq*® PCR SuperMix (TransGen Biotech, China), 1x. Sterilised distilled water was used to mark up the total volume.

Next, the PCR tubes were placed into thermocycler Eppendorf, Vapo.project, USA. The PCR protocol was set up as follow; Initialisation, 94°C for 5 min; Denaturation, 94°C for 30 sec; Annealing, 55°C for 30 sec; Extension, 72°C for 90°C sec; Final extension, 72°C for 10 min and lastly; Final hold at 10°C for indefinite. The denaturation, annealing and extension reaction were repeated for 35 cycles. After the PCR was completed, PCR products were loaded into 1% (w/v) of 1x TAE agarose gel. Prior loading, the PCR products were mixed with 1 µL of loading buffer. A volume of 2 µL of 1 Kb DNA marker was used as standard. Electrophoresis was done at 80V for 2 h. After completed, gel imaging was captured by gel documentation machine.

### *3.6 Preparation of Glycerol Stock*

A single colony of respective *E. coli* strains were inoculated into LB and incubated overnight at 200 rpm, 37°C. Recombinant cultures were cultivated with ampicillin at concentration of 100µM and 50µM for pTrcHis and pUC8.2-14, respectively. The cultures were centrifuge at 10 000 x *g* for 2 min. Ten percent of the culture were transferred into fresh LB medium and incubated at 200 rpm, 37°C until the optical density (OD) at 600nm achieved 0.6. Once the OD was achieved, the cultures were centrifuge at 10 000 x *g* for 2 min. Supernatants were discarded and 250 µL of fresh LB was added into the centrifuge tubes. Glycerol stock solution was added into the culture until final concentration of 25% (v/v). The cultures were gently mixed and kept in -80°C for future use.

### 3.7 *Preparation of Inoculum*

A single colony of *E. coli* was transferred into sterile Luria Bertani broth (LB) with 10 g/L glucose. The glucose was prepared separately prior autoclaving. Filtered sterile ampicillin with respective concentrations for *E. coli* carrying pTrcHis and pUC8.2-14 was also added into the broth. The incubation was agitated for 250 rpm at 37°C for 12 hours.

### 3.8 *Preparation of Shake Flask Cultivation*

Ten per cent of inoculum was added into the flasks with fresh sterile LB broth with 10 g/L of glucose. Ampicillin was also added to final concentration of 100 µM and 50 µM for pTrcHis and pUC8.2-14 respectively. The flask was incubated with agitation of 250 rpm at 37°C. Samplings of 3 mL were taken every hour for 12 hours.

### 3.9 *Preparation of Batch 2 L Stirred Tank Bioreactor (STB)*

Prior autoclaving, the 2 L STB Biostat® A Plus Sartorius with BioPAT® MFCS/DA 2.1 was calibrated with 100% nitrogen gas and pH buffer of 7 and 4 accordingly. LB broth, ampicillin and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were prepared accordingly. In batch cultivation, glucose was supplied as carbon source to the culture. Ten per cent of inoculum was inoculated into the calibrated system and the data acquisition was started to collect data from bioreactor's parameters (agitation speed, DO, pH, temperature, amount of acid and base). IPTG was induced as described elsewhere in the study.

### 3.10 *Preparation of Fed-batch 2-L STB*

A 2 STB was prepared for accordingly to previous specification prior to the fed-batch cultivation mode. The fed-batch cultivation was started when the carbon source in the batch culture was found depleted. The feeding medium was sterilised and aseptically introduced to the *E. coli* culture. The cultivation was cultivated up to 72 h and samplings were taken periodically.

### 3.11 *Preparation of 2-L STB Integrated with Ultrasonic System*

A 2 L STB was prepared accordingly to previous specification. In addition, a 20 kHz, 600 W maximum power, Misonix Sonicator® 3000 (Misonix, Inc. USA) US generator was used in combination with a standard tapped sonic horn (Misonix, Inc., part no. 200 with 12.7 mm tip diameter, 127 mm length), or sonotrode, installed in an external 800B Misonix Flocell® with a diameter inlet orifice. The horn had a replaceable flat tip made of titanium alloy (Misonix, Inc., part no. 406). The flow cell, with the sonic horn in place, was autoclaved (121°C, 20 min) cooled to room temperature, and connected to the bioreactor aseptically using sterile silicone tubing. The medium from the bioreactor was recirculated continuously through the sonic chamber using a calibrated peristaltic pump (Masterflex, USA). The recirculation flow rate was fixed at 0.2 L/min. The recirculation commenced after the fermenter had been inoculated and briefly mixed. All the fermentations were carried out with recirculation of the broth through the sonic chamber, but US was not applied in the control fermentations. For fermentations that were undergo sonication, the sonication was commenced at specified conditions only after 9.5 h of the inoculation of the bioreactor. The fermentation setup was displayed in Figure 1.

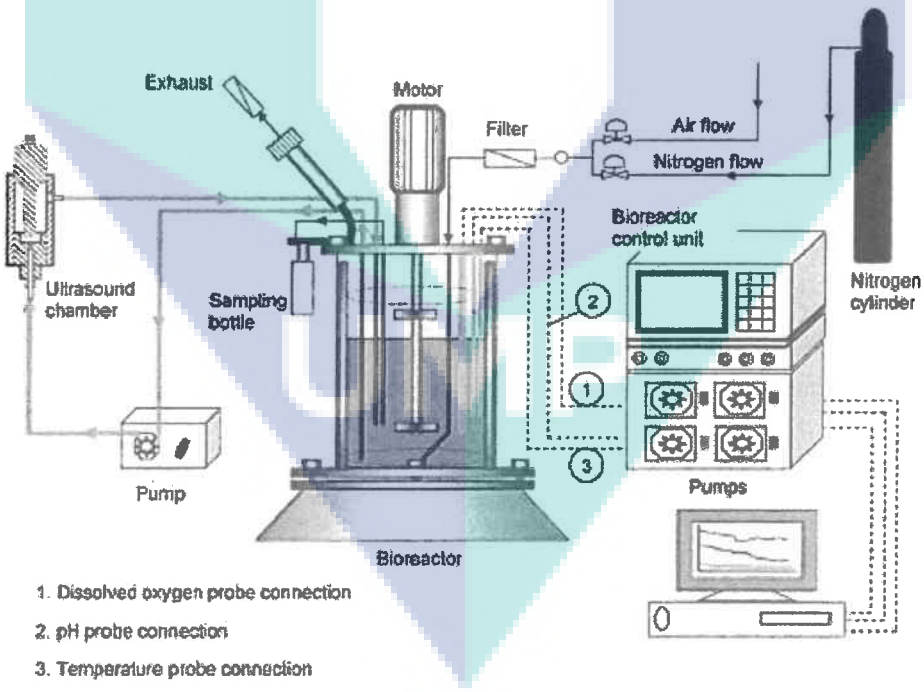


Figure 1. 2 L stirred tank bioreactor arrangement with ultrasound system (adapted from Sulaiman, Ajit [10] with permission).

### 3.12 *Power Number Determination*

For the power number determination, Meusel, Loffelholz [11] suggested the use of torque sensor to measure the specific power input, ($P/V_L$). Due to limited tools in this study, the power number was determined based on the geometrical features.

### 3.13 *Mixing Time Determination*

The mixing time was obtained by using iodometry and conductivity method as mentioned in Meusel, Loffelholz [11]. In the iodometry methods, iodine potassium iodide (0.1 N) was added with starch solution (2 $gL^{-1}$) until the colour was completely changed to dark blue (approximately after 2 minutes). The bioreactor was set at 500 rpm and a video recorder was set for recording. Once sodium thiosulfate solution (0.1 N) was added in the vessel, the timer was also started. The time measurement was stopped when the colour change from dark blue to colourless was achieved. The solution was exchanged after each reaction for at least triplicates readings.

For the conductivity method, a multi-parameter bench meter, Eutech PC2700, Eutech Instrument was installed on the top of the vessel plate. At constant room temperature, the vessel was filled with ultrapure water type 2. The agitation was run at 500 rpm and waited approximately 2 minutes to allow a quasi-stationary fluid flow pattern. The data acquisition was started once the conductivity solution, 4 M KCl was added into the vessel. The measurement was complete after a stable reading had achieved. The solution was exchanged after every three measurements.

### 3.14 *Response Time Determination*

This method was found in Meusel, Loffelholz [11] and Scargiali, Busciglio [12]. The bioreactor was filled with Phosphate Buffered Saline (PBS) solution and the DO probe was polarised at least 6 h. Prior the measurements were conducted, the probe calibration was performed with nitrogen and oxygen until 0 % and 100 % were achieved,

respectively. The following parameters were set for the calibration, 1.36 m/s $\mu_{Tip}$, maximum nitrogen aeration $ß_{N2}$, and 1 vvm $ß_{Air}$.

The response time of the DO probe was measured by using PBS saturated with nitrogen (beaker A) and PBS saturated with oxygen (beaker B). The DO probe was covered in beaker A until constant reading was achieved. Next, the probe was put in beaker B and data acquisition was recorded until constant value was achieved. The measurement was taken in at least triplicate to measure the response.

### *3.15 Volumetric Mass Coefficient via Gassing-out Method*

The bioreactor setup was similar as previous. The agitation was set at 500rpm. The vessel was sparged with nitrogen until it was saturated. The measurement was started once the air was supplied and the reading of oxygen became stable or saturated at 100 %. The measurements were taken triplicates.

### *3.16 Sample Treatment and Scanning Electron Microscopy (SEM)*

The sample treatment procedure was adapted from Microscopy Unit (Electron Microscopy), Institute of Bioscience, UPM. The coated samples were brought to viewing with Jeol JSM6400, USA. The beam current used was 15 kV and working distance was between 15 to 21 mm.

# 4.0 LITERATURE REVIEW

Ultrasound is sound wave of frequency higher than the audible range of human hearing capability. Normal human hearing range is approximately 20kHz whereas the ultrasound frequencies can be found from 20kHz to gigahertz. The application of ultrasound is various in many industries such as transportation, oil and gas, medical and food manufacturing. In bioprocessing involving microorganism cultivation, the use of ultrasound was found to employ two types of ultrasound, ultrasonicator probe and ultrasonicator bath tank. The effect of using minimize or inactivation of cells, which as shown by Herceg, Jambrak [13]. Cultivation of *K. marxianus* by Sulaiman, Ajit [10] also showed a decrease in the cell's viability but an increase for the aimed bioproduct, ethanol at 20% duty cycle.

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 [14, 15], the increasing demands of lipases are shown by many productions from wild-type [16, 17] or genetically modified organisms [18-21]. 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 [22]. 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 [23], more strategies are needed to enhance the cultivation of the individual expression hosts. Among the current strategies are strain phenotype improvement [24], high throughput technology [25], and feeding strategy [26]. 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 [13, 28, 29], inactivation of enzymes and pathogens to avoid food spoilage [13], and assisting in enzymatic reaction towards insoluble substrate [30]. Owing to the potential of US in microbiology, some of the US parameters have been introduced in bioprocesses improvement from shake flask culture [31] to a laboratory bioreactor [1, 6, 10]. In this study, the use of US towards the small-scale cultivation of recombinant culture, particularly using 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.

Many expression hosts from *Escherichia coli* derivatives were used in harbouring recombinant protein. *E. coli* was known as well studied and fast-growing bacterium among other species and potentially harvested up to 100 g of dry cell weight per L. One of common use as an expression host is *E. coli* BL21 (DE3). Many studies have been done in detail about BL21 (DE3) [32], the ancestors and relatives [33], and proven previous research in the production of protein expressions [34-37]. Rosano and Ceccarelli [35] also suggested two strains of *E. coli*, BL21 (DE3) and some derivatives of K-12 lineage in the first screening. BL21 (DE3) was recorded to be missing of two genes which are contributing to the better performance of the protein expression, Lon protease [38] and OmpT [39]. By the result of thorough justification and previous studies, thus BL21 (DE3) was chosen in this study to be the expression host for the recombinant plasmids of lipase production.

# 5.0 FINDINGS

## *5.1 Concentration and Plasmids Purity*

The extracted plasmids from *E. coli* Top 10 pTrcHis, *E. coli* ATCC®68046™ pUC8.2-14 and the respective transformants, *E. coli* BL21 (DE3) pTrcHis, and pUC8.2-14 were quantified to evaluate the plasmid concentration in the respective cultures. High concentration and ideal purity of plasmids were required to perform DNA sequencing. From three replicates of cultures measured, the concentration and purity of pTrcHis and pUC8.2-14 from different hosts were obtained as shown in Table 1.

Table 1: Concentration and purity of pTrcHis and pUC8.2-14 from different hosts.

| **Host** | ***E. coli* Top 10** | ***E. coli* ATCC® 68046™** | ***E. coli* BL21 (DE3)** | |
|---|---|---|---|---|
| **Plasmid** | pTrcHis | pUC8.2-14 | pTrcHis | pUC8.2-14 |
| **Concentration at 260 nm (ng/µL)** | 67.13 | 64.5 | 131.5 | 192.5 |
| **Ratio $A_{260/280}$** | 1.98 | 1.67 | 1.865 | 1.851 |
| **Ratio $A_{260/230}$** | 2.25 | 0.97 | 2.156 | 2.305 |

From Table 1, the concentrations from donor plasmids were lower and less purity namely the *E. coli* ATCC®68046™ pUC8.2-14. Hence, for the transformants, high concentration and a better ratio of purity were gained by a collective of four tubes of extracted plasmids and repurification by GeneAll® Expin™ Combo GP, GeneAll, Korea. Later, both plasmids from the transformants showed high concentration and ideal ratio of purity as suggested by Held, 2001 [40]. The absorbances used from the spectrometry were 260nm for nucleic acids, 230nm for phenolic compounds and polysaccharides while 280 nm for protein contaminants. The ideal purity $A_{260/280}$ of any extracted DNA plasmid is the ratio between 1.8 to 2.0, and higher ratio indicating of RNA contamination. Ideal purity $A_{260/230}$ should be more than 2.0, representing higher DNA products over

protein contaminants. Based on the high concentration and ideal purity of the respective ratios, both plasmids were accepted for observation on gel electrophoresis and DNA sequencing.

## *5.2 PCR Products*

From the 1% (w/v) agarose gel in Fig. 2A, the plasmid and PCR products of the respective plasmids were shown present, except for pUC8.2-14 in lane four. The absence was due to low concentration in loading the samples for plasmid which was 1 µL for each while 5 µL for PCR products. Loading was then increased to 5 µL for plasmid in Fig. 2B and thus the plasmids were exhibited at approximately 4 kb and 3 kb respectively for pTrcHis and pUC8.2-14 (indicated with the red circles at lane seven and eight). The PCR products of both lipase genes were obtained approximately at 1.2 to 1.3 kb. The pUC8.2-14 was a match expectedly as described by the previous researcher in the ATCC repository [41]. While for *Staphylococcus hyicus*, there were two groups of nucleotide registered on NCBI, *Staphylococcus hyicus sp hyicus* [42] and *Staphylococcus hyicus* ATCC11249. Both groups were designated to lipase genes.

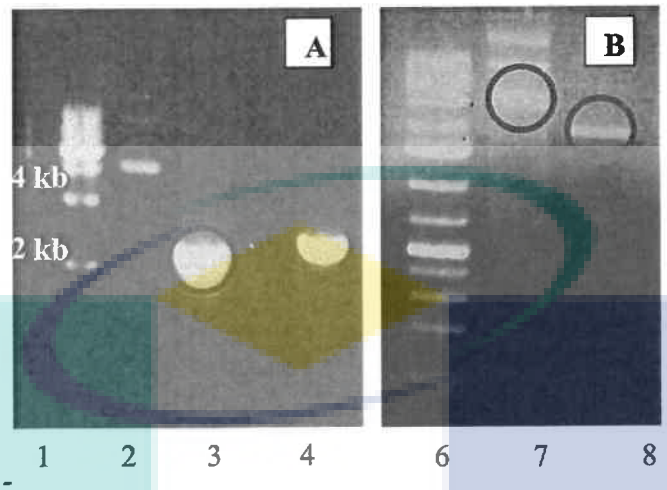


Figure 2. Plasmids and PCR products of respective pTrcHis and pUC8.2-14. Lane 1-1 kb DNA marker, Lane 2- pTrcHis; Lane 3- pTrcHis PCR product; Lane 4- pUC8.2-14; Lane 5- pUC8.2-14 PCR product; Lane 6 – 1kb plus DNA marker, Lane 7- pTrcHis and Lane 8- pUC8.2-14. The red circles were showing the respective plasmids and PCR products.

### 5.3 *DNA Sequencing Analysis from NCBI Databases*

DNA sequencing obtained from the sequencing service was uploaded into BLAST using the NCBI website. From the non-redundant databases, the respective plasmids were found to be 99% and 47% query cover for pTrcHis and pUC8.2-14 respectively. The pTrcHis alignment was confirmed as *Staphylococcus hyicus* strain ATCC11249 and lip gene for lipase [42]. In a recent study, ATCC11249 was also being analysed for exudative epidermis of swine [43]. Meanwhile, pUC8.2-14 alignment was found containing fragment from *Rhizopus delemar* carboxyl ester hydrolase mRNA, *Rhizopus niveus,* and *Rhizopus oryzae* gene for lipase [41].

### 5.4 *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 2). 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, Anurag [44].

Table 2. Coded and actual values of the factors.

| Factor[a] | 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** |

[a] *centre point is the average of minimum and maximum*

### 5.5 *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 Δ, %), 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 Fig. 3. According to the two top graphs in Fig.3, the perpendicular lines clearly projects the interaction of the high level and low level of the amplitude and duty cycle. The same pattern is demonstrated by duty cycle and treatment time. Hence, both interactions are significant while other factors kept at their constant values. Moreover, in the two bottom graphs in Fig.3, 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 [45]. However, from Equation (1), 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.

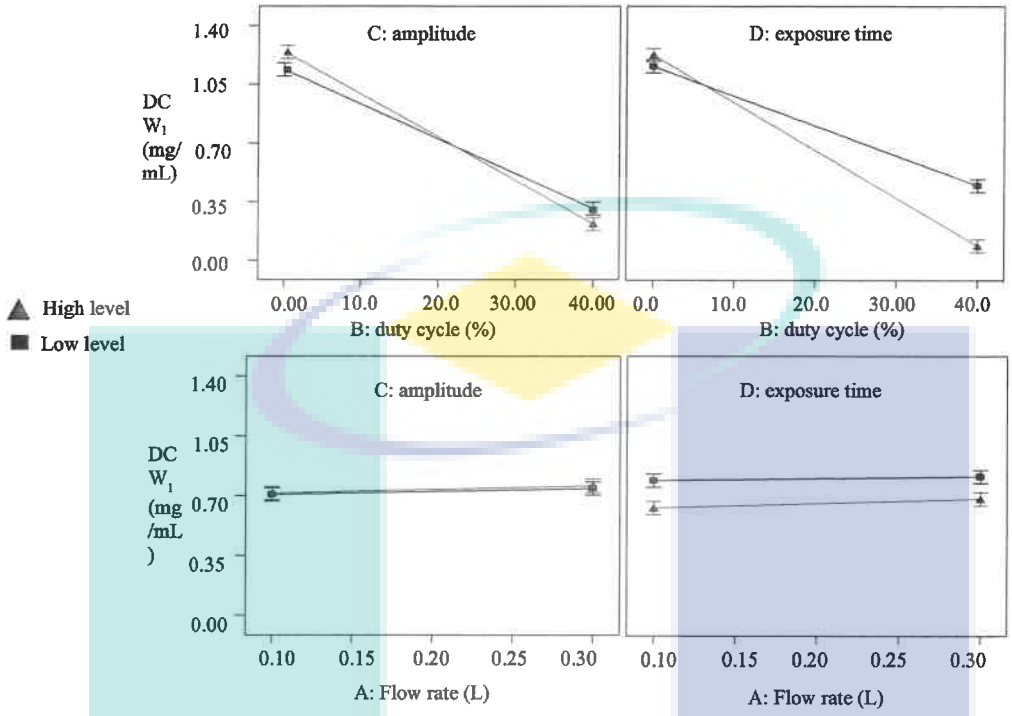


Figure 3. Interaction studies between selected variables for response DCW.

$$DCW_1 = +0.73 + 0.019A - 0.46B + 6.369E^{-003}C - 0.073D - 3.019E^{-003}AB + 1.719E^{-003}AC + 7.256E^{-003}AD - 0.047BC - 0.11BD + 9.444E^{-003}CD + 2.919E^{-003}ABC - 0.023ABD + 0.017ACD + 0.016BCD \quad (1)$$

### 5.6 *Effect of main factors and interactions for lipase*

From Table 3, the ANOVA shows that the effect of factors at any term or interaction for the lipase activity are not significant. A negative predicted $R^2$ 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 posttreatment time of sonication towards the lipase activity. The normality test by Shapiro-Wilk has passed at P=0.777 [46]. 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 posttreatment, (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 DCW.

### 5.7 *Profiles of sonication outputs*

Four sonication outputs are summarised in Fig.4. 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 [47]. 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".

Table 3. Three-way interaction ANOVA for DCW of recombinant E. *coli.*

| | DCW | | | |
|---|---|---|---|---|
| Source | Sum of squares | Mean square | *F* value | *P*-value |
| Model | 3.73 | 0.27 | 123.69 | < 0.0001[a] |
| A | 5.92E-003 | 5.92E-003 | 2.74 | 0.1487 |
| B | 3.41 | 3.41 | 1579.33 | < 0.0001[a] |
| C | 6.49E-004 | 6.49E-004 | 0.30 | 0.6031 |
| D | 0.08 | 0.08 | 39.18 | 0.0008[a] |
| AB | 1.46E-004 | 1.46E-004 | 0.068 | 0.8035 |
| AC | 4.73E-005 | 4.73E-005 | 0.022 | 0.8872 |
| AD | 8.43E-004 | 8.43E-004 | 0.39 | 0.5550 |
| BC | 0.04 | 0.04 | 16.37 | 0.0068[a] |
| BD | 0.18 | 0.18 | 84.37 | < 0.0001[a] |
| CD | 1.43E-003 | 1.43E-003 | 0.66 | 0.4471 |
| ABC | 1.36E-004 | 1.36E-004 | 0.063 | 0.8099 |
| ABD | 8.66E-003 | 8.66E-003 | 4.02 | 0.0919 |
| ACD | 4.87E-003 | 4.87E-003 | 2.26 | 0.1837 |
| BCD | 3.95E-003 | 3.95E-003 | 1.83 | 0.2245 |
| Curvature | 0.72 | 0.72 | 333.28 | < 0.0001[a] |
| Residual | 0.01 | 2.16E-003 | | |
| Lack of Fit | 4.32E-003 | 4.32E-003 | 2.51 | 0.1743 |
| $R^2$ | | 0.9961 | | |
| Adjusted $R^2$ | | 0.9871 | | |
| Predicted $R^2$ | | 0.4789 | | |
| Adequate Precision | | 31.693 | | |
| Coefficient of Variance % | | 0.9961 | | |

A-Flow rate, B-duty cycle, C-amplitude, D-treatment time,
[a] – significant value of $p<0.05$

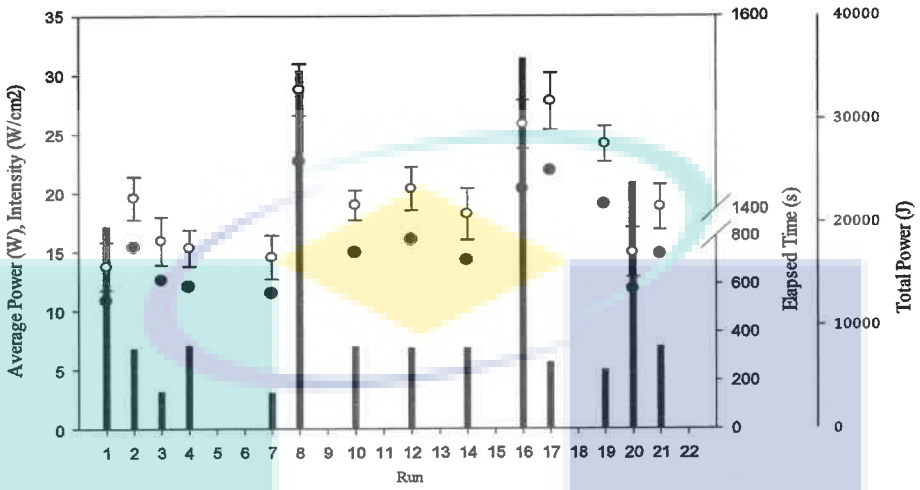


Figure 4. Sonication output profiles from experimental run, elapsed time ( ■ ), total power ( ■ ), average power ( ○ ), and sonication intensity ( ● ).

The rapid shift of sonication increased the intensity and with longer treatment time, the cells were damaged [13]. In this study, only two main factors and two interactions are significant as described earlier in Table 3. In addition to the screening factors, a Pareto chart is used to illustrate the importance of the factors complete with Bonferroni correction [49]. Therefore, Fig.5 shows a Pareto chart of response coefficient for $DCW_1$ and lipase activity. Fig.5a describes that the factors above Bonferroni level are significant according to the positive and adverse responses, while Fig.5b shows that all the factors are below the t-value limit.

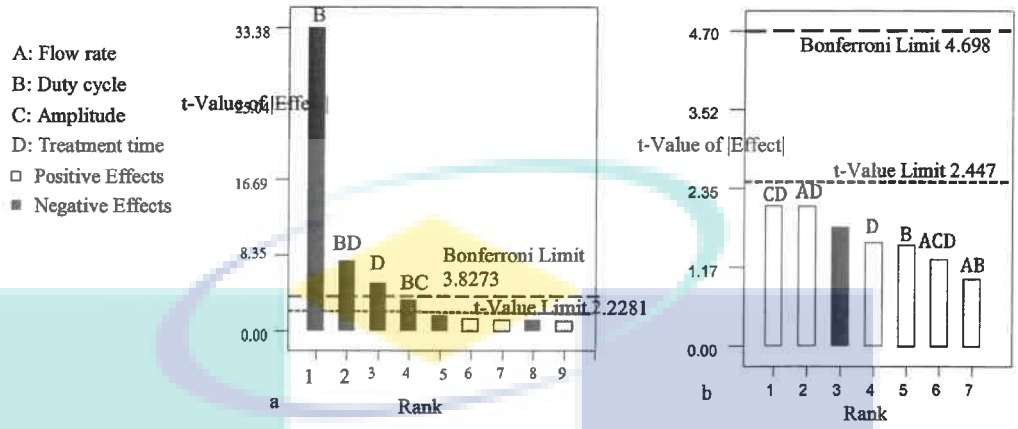


Figure 5. Response coefficient significance study on DCW (a), and lipase (b).

## 5.8 *Validation of FFD*

The validation experimental design was recommended by the software 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.

## 5.9 *Power Number*

The power number was generated by comparing the calculated value against given information on Reynold number ($N_{Re}$) to power number in McCabe, Smith [50].

The Reynold number is using formula as follows,

$$N_{Re} = Da^2.n.d.u^{-1}$$ Eq. 2

Where,

Da is the diameter of impeller, 0.052 m

n is the agitation speed used, 8.33 rps

d is the density of liquid, 1000 $kg.m^{-3}$

u is the viscosity of liquid, 0.0009 Pa.s

From the setting, the $N_{Re}$ was calculated to be 25, 027. Based on the figure 10.59 by Uhl and Grey (1967) in Sinnott [51], the curve #3 was selected as similar to the current setting. The figure suggested, the $N_P$ was about ~3.

In other calculation, using a formula given in the BioFlo115 manual [52], they approximate the use of horsepower (HP) for the bioreactor unit as follows

$$HP = D^5.RPM^3.(4.5\,x10^{-13}).I$$ Eq. 3

Where,

*HP* is the horsepower,

*D* is the impeller, 2.047 in inches,

*RPM* is the agitation speed, 500rpm

*4.5 x* $10^{-13}$ is the constant factor based on unaerated water at 20°C with a six-bladed Rushton impeller,

*I* is the number of impeller use; 1 for one impeller, 1.8 for two impellers, 2.4 for three impellers

Based on the above information, the HP was calculated 0.05 N.m. Thus, the power can be calculated from the HP, 0.05 N.m using the following formula,

$$P = 2\pi nT \qquad \text{Eq. 4}$$

Where

$n$ is the rotation per seconds, 8.33 rps,

$T$ is torque, 0.05 N.m

The power obtained was 2.617 kW.

### *5.10 Mixing Time*

The iodometry method was evaluated by the decolourisation of iodine-starch solution to colourless. From the observation in Fig.6, the colourless solution was achieved after two or three seconds of the sodium thiosulphate addition regardless the experimental settings. The impeller tip speed used for the measurement was constant at 1.36 m/s.

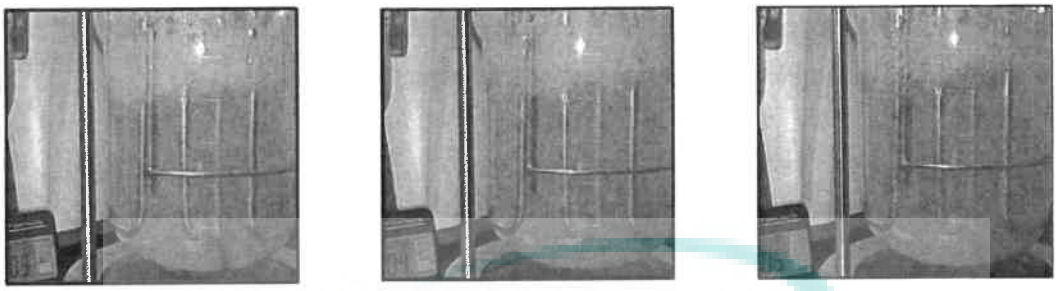

Figure 6: The colour exchange in iodometry method.

In the conductivity method, the readings were determined for maximum and minimum conductivity value, $\kappa_\infty$ and $\kappa_0$, respectively. Homogeneity ($H_0(t)$) was calculated based on the following equation 4, and next the homogeneities were plotted as function of time on a line plot in figure 7.

$$H_0(t) = \frac{K(t) - K_0}{K_\infty - K_0} \qquad \text{Eq. 5}$$

The mixing time was determined by identifying the time (s) that crosses the line of 0.95 (95%) or 1.05 (105%) homogeneity at the last time. The mixing time values were averaged by the arithmetic average using equation 6.

$$\theta_{(RT)} = \frac{1}{n}\sum_{i=1}^{n}\theta_i \frac{\theta_1 + \theta_2 + \theta_3 \ldots + \theta_n}{n} \qquad \text{Eq. 6}$$

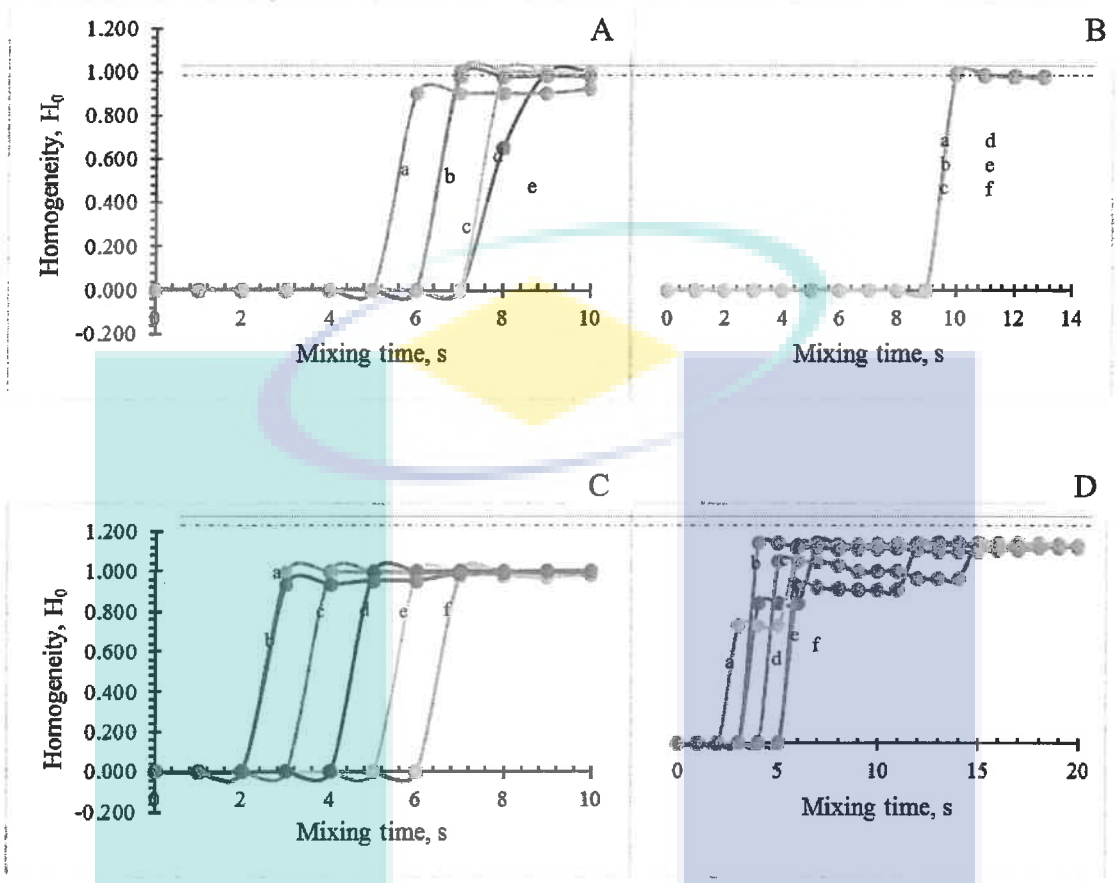


Figure 7 Conductivity assessment, A – control, B – flowrate at 0.2 $Lmin^{-1}$, C – 10% duty cycle, D – 40% duty cycle.

### *5.11 Response Time*

In this study, 21 s was recorded to achieve 95% DO. Polarographic electrodes can achieve 95% DO in 0.05- 15 s [53]. Response at 63% DO ($t_{63\%}$) takes 8 s as shows in Fig.8. A maximum response time of $t_{63\%}$ is recommended at $t_{63\%}$ < 30 s [11].

### 5.14 Growth Profiles

Figure 11 shows the sonicated DCW was found higher than the control, induced DCW. The induced culture was showing slow growth rate due to the production of the recombinant protein. Similar profiles by Qingbao, Ling [54]. Glucose consumption was higher in sonicated compared to the control, induced. Acetic acid was found present in both cultivations at considerably low concentration which is not at the inhibiting level [55]. SEM images of damaged cells after 10% duty cycle treatment (Fig.12). From the same cultivation setup, the expression of lipase at ~ 42 kDa were visualised after induction of 1 mM IPTG (Fig. 12C). The expression of lipase was found little after sonication.

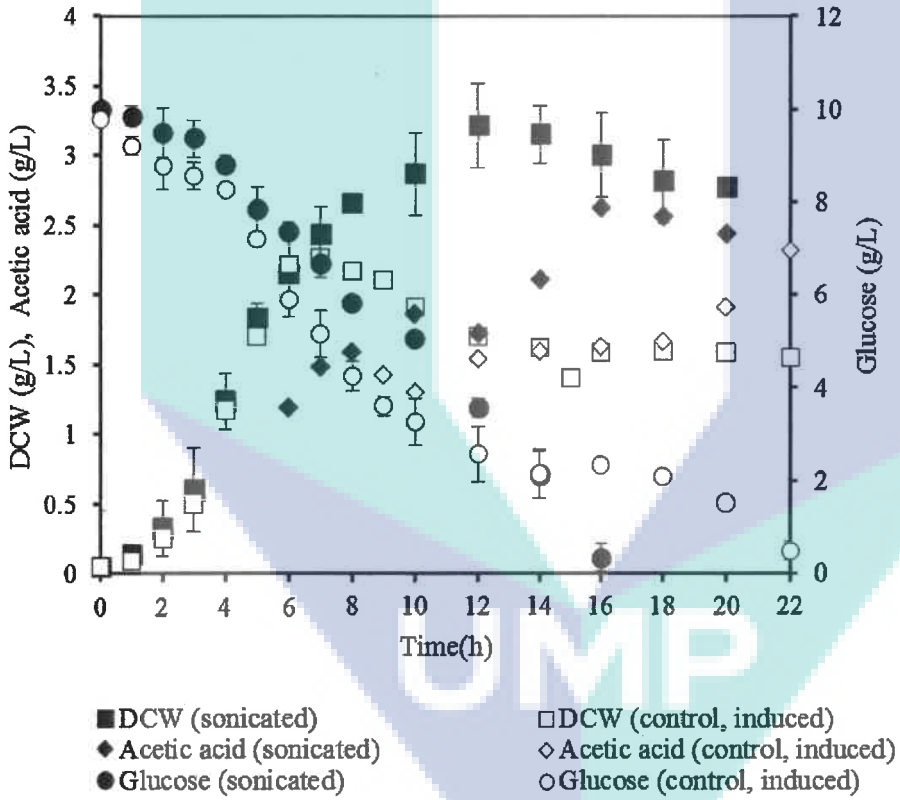


Figure 11. The growth profile of recombinant lipase in 2-L sonobioreactor.

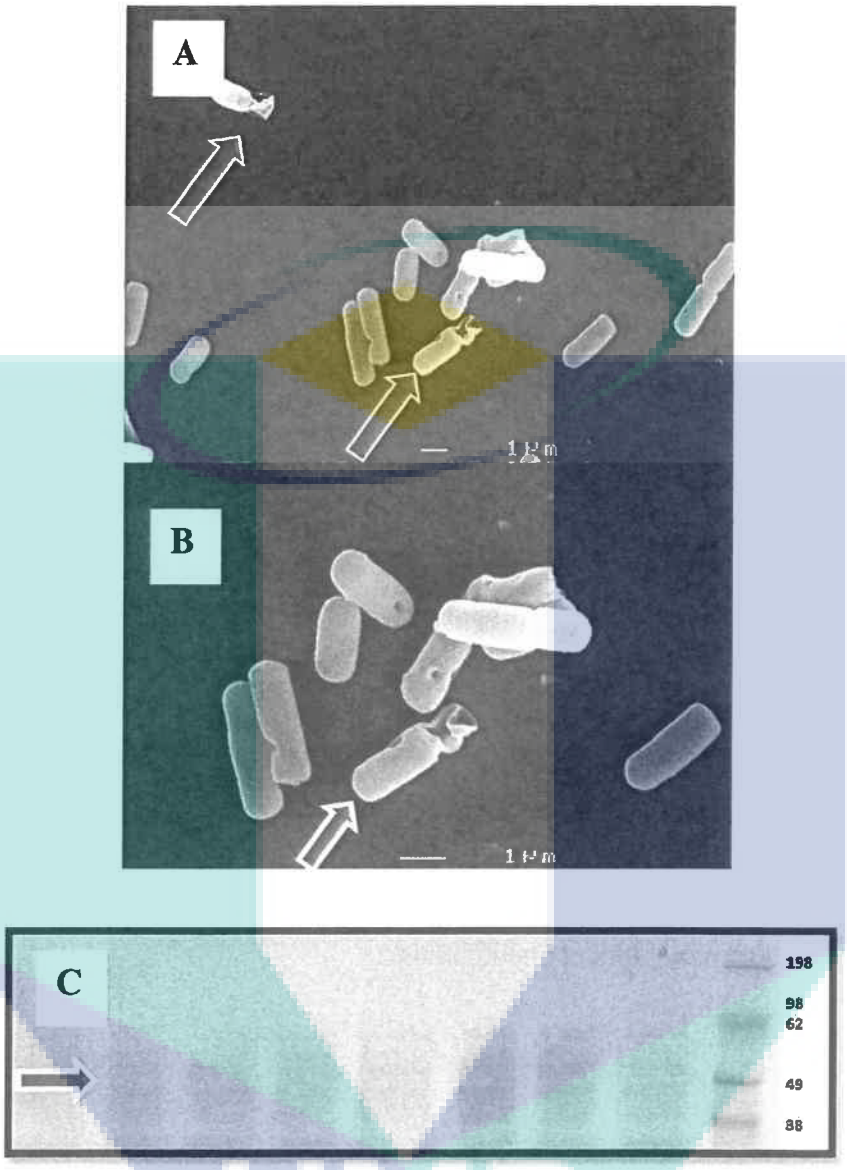


Figure 12. A and B are the SEM images of recombinant *E. coli* after treatment with 10% duty cycle sonication. C is the SDS-PAGE for the total protein with very low lipase expression.

A – magnification at 5K, B – magnification at 9K, focuses on the cell destruction.

# 6.0 CONCLUSION

Bioprocess characterisation was able to determine by using experimental and calculation via given formulas. Batch fermentation of control (non-sonicated) against the sonicated cultivation at 10% duty cycle were compared by the condition's profiles such as pH, DO, agitation speed, temperature. The recombinant growth profiles only discussed on dry cell weight, glucose consumption and acetic acid. Other by-products, like lactic acid and ethanol were absent throughout the cultivation. The cells were found degraded at 10% duty cycle, thus predicting more impact when using higher sonication.

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.

## 7.0 ACHIEVEMENT

i) Name of articles/ manuscripts/ books published & Title of Paper presentations (international/ local)

1. Title: Bioprocesses Characterization of 2-L Sonobioreactor

*Wan Siti Atikah Wan Omar, Ahmad Ziad Sulaiman, Azilah Ajit*
The 3rd International Conference on Green Chemical Engineering Technology (3rd GCET_2017): Materials Science
07-08 November 2017, Universiti Kuala Lumpur

2. Title: Effect of flow rate, duty cycle, amplitude, and treatment time of ultrasonic regimens towards *Escherichia coli* harbouring lipase

*W S A W Omar, A Z Sulaiman A Ajit, Y Chisti, A L T Chor*
29th Symposium of Malaysian Chemical Engineers (SOMChE) 2016
Curtin University, Miri Sarawak (Malaysia Campus)

3. Title: Transformation of pTrcHis and pUC8.2-14 into Escherichia coli BL21 (DE3)

*Wan Siti Atikah Wan Omar, Ahmad Ziad Sulaiman, Azilah Ajit, Yusuf Chisti, Mohd Hairul Ab Rahim, Adam Leow Thean Chor.*
The Third National Conference for Postgraduate Research (NCON-PGR2016)
Universiti Malaysia Pahang

ii) Human Capital Development

1. PhD. - WAN SITI ATIKAH WAN OMAR - PKB14006
2. MSc. - NOORHAFIZA BINTI YAHYA - MKT17007
3. MSc. NORNASUHA BINTI ABDULLAH - MKC16030

iii) Awards/ Others

International Festival of Innovation on Green Technology (i-FINOG 2018)
Wan Siti Atikah Wan Omar, Nurhafizah Ibrahim, Ahmad Ziad Sulaiman, Azilah Ajit
Silver Medal
20-22nd April 2018, Universiti Malaysia Pahang

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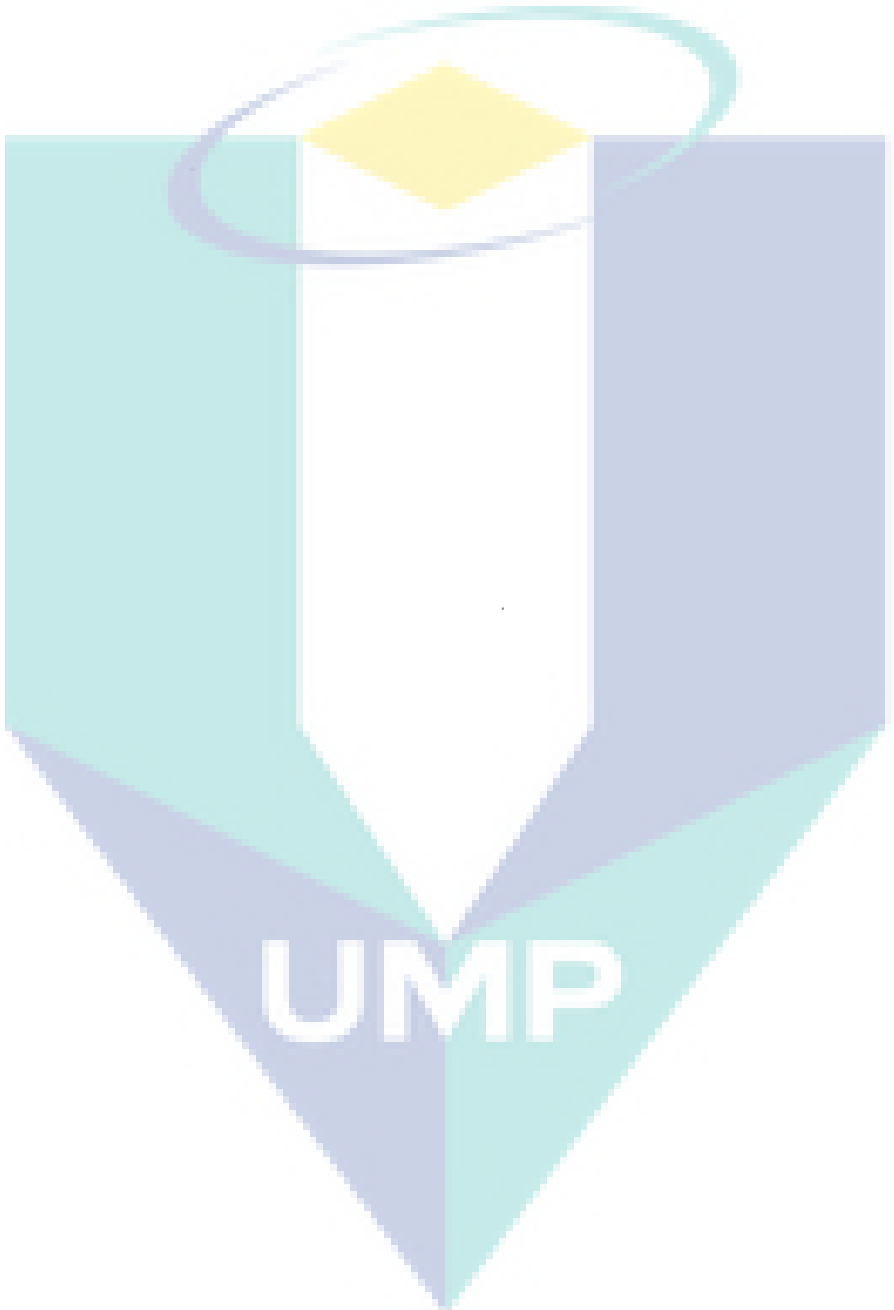
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