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# FATTY ACID AS THE POTENTIAL INDUCER FOR RECOMBINANT LIPASE EXPRESSION IN *Meyerozyma guilliermondii* STRAIN SO

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**Abstract**

High amount of methanol concentration in the cultivation medium during the production of enzyme in methylotrophic yeast could inhibit the cell growth, level of enzyme expressed as well as limit the use of the enzyme for certain foods and pharmaceutical production. A recombinant T1 lipase was expressed using *Meyerozyma guilliermondii* strain SO as host under the regulation of alcohol oxidase promoter without methanol induction. Thus, this study aimed to decipher an alternate innate inducer of the expression host by determining the metabolites present in the recombinant strain SO and investigate the expression of the bacterial lipase using the significant selected metabolite (fatty acids). The media from *M. guilliermondii* strain SO (wild type) and SO2 (recombinant strain) (at 0 and 60 h) were extracted using methanol extraction protocols followed by gas chromatography-mass spectrometry (GC-MS). A multivariate statistical analysis; principle component analysis (PCA) and partial least square discriminant analysis (PLSDA) were implemented to determine the relationship between the metabolites present in strain SO and SO2. The results showed that the primary metabolites; amino acids, organic acids and particularly, copious amount of fatty acids, were significantly present in strain SO2 compared to strain SO. Further analysis on the identified fatty acids were conducted and the results showed that hexadecanoic acid (C16) showed an increase of 1.45 fold of T1 lipase expression in SO2 compared to the control experiment. This finding suggested that the fatty acid could be used as an alternative inducer for T1 lipase expression to reduce and/or eliminate the application of methanol.

## INTRODUCTION

In recent times, metabolomics has been explored as a systematic and quantitative assessment tool to comprehend biological samples [1]. The study of the metabolome gives an instantaneous fingerprint of the physiology of a cell and very useful information about biological processes [2]. Metabolomics plays an important role in connecting the

phenotype and genotype gaps, since it magnifies the modifications in the proteome and provides a better representation of the phenotype of an organism [3]. Gas chromatography-mass spectrometry (GC-MS) is gradually becoming an important tool to characterize the metabolites in a complex biological system. In recent study, several hyphae-inhibiting metabolites and a biofilm-forming metabolite-tyrosol, have been successfully profiled using GC-MS in *Candida auris* [4].

In recombinant gene technology, other than the prokaryotic expression system, yeasts have been reported to be an expression host. Commonly used non-conventional yeast such as *Komagataella phaffii* (previously known as *Pichia pastoris*), has been recognized as a cost-effective and valuable host for the production of heterologous proteins because of its major advantages over prokaryotic expression systems [5]. *M. guilliermondii* is a model organism for flavinogenic yeasts. It has a high reputation to over-synthesis the riboflavin during starvation of iron [6].

*M. guilliermondii* strain SO was first isolated from spoiled orange by Oslan *et al.,* [7]. In 2015, Oslan *et al.,* [8], conducted the first study on the ability of *M. guilliermondii* strain SO as a host to express thermostable T1 lipase from *Geobacillus zalihae* using pPICZαB vector consisting of alcohol oxidase promoter ($P_{AOX1}$). Later, Abu *et al.,* [9] reported on the ability of *M. guilliermondii* strain SO to express the recombinant protein in the absence of methanol with 2 U/ml in buffered medium. The question that arose was, what caused the recombinant T1 lipase to be expressed without methanol induction? Generally, $P_{AOX1}$ regulation in *K. phaffii*, requires methanol to initiate the transcription of the recombinant protein. This is also proven by a recent study, where methanol is vital for $P_{AOX1}$ regulation [10]. In 2014, T1 lipase was overexpressed in *K. phaffii* using similar vector pPICZαB. The optimum methanol induction used was 1.5 % (v/v) for 192 h cultivation [11]. No significant lipase expression was observed in the absence of methanol as compared to *M. guilliermondii* strain SO.

Therefore, this research aimed to use the metabolic profiling data to identify the compounds that might cause T1 lipase to be expressed in *M. guilliermondii* strain SO without methanol induction. The metabolic profiling of the intra- and extracellular environment of recombinant T1 lipase strain SO was conducted using untargeted metabolites. Then, the most abundant compounds present in the recombinant strain were used to investigate the effect on T1 lipase expression.

## MATERIALS AND METHODS

### Strains, Media and Culture Conditions

Wild-type *M. guilliermondii* strain SO and its recombinant SO/pPICZαB/T1 lipase (SO2) were obtained from the previous study [8]. The wild-type strain SO was streaked on yeast peptone dextrose (YPD) agar plate [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar], while recombinant SO2 was streaked on YPD agar supplemented with 100 µl $ml^{-1}$ Zeocin, incubated at 30°C for 3 days. Then, a single colony was inoculated into 10 ml YPD broth and incubated overnight at 30°C with 250 rpm agitation. Next, 1% (v/v) culture was inoculated into 100 ml of complex YPTG medium [Yeast Peptone Tryptic Glycerol; 1% (w/v) yeast extract, 2% (w/v) peptone, $4 \times 10^{-5}$% (w/v) biotin, 0.2% (w/v) tryptic soy broth, 1% (v/v) glycerol] in 500 ml conical flask at 30°C, 250 rpm agitation for 24 h and the growth OD at 600nm was monitored. The initial $OD_{600nm}$ ~ 15 was used for induction in 100 ml YPTM medium [similar composition as YPTG where glycerol was substituted with 0.5% (v/v) methanol and YPT medium (YPTM composition without methanol)]. Then, the cell was cultivated for 120 h and YPTM was supplemented with methanol every 24 h interval. Lipase activity and cell growth were determined for each 12 h intervals. After the optimum time was determined, 5 ml culture was harvested at 0 h and 60 h from cultures incubated in YPT medium for metabolite extraction both intra and extracellular environments.

### Lipase Activity Determination

The lipase activity was determined *via* colorimetrical assay described by Kwon and Rhee [12]. The substrate emulsion was prepared by mixing together to an equal volume of 1:1 (v/v) of olive oil (Bertolli, Italy) and glycine-NaOH buffer (pH 9) with a homogenizer. Then, the enzyme was assayed at 70°C according to Leow *et al.,* [13] where one unit (U) of lipase activity is defined as the rate of fatty acid formation in µmole per min under standard assay condition.

### Sampling, Quenching and Extraction of Extracellular Metabolites

The collected 5 ml samples were centrifuged at 5000 ×*g* for 1 min at 4°C. The clear supernatant was transferred into a clean falcon tube. The supernatant was then separated into 1 ml aliquots followed by the addition of distilled water (top up to 10 ml). Then, the samples were freeze dried under low temperature and stored at −80 °C for further analysis. Each extraction was performed in three biological replicates and three technical replicates.

### Sampling, Quenching and Extraction of Intracellular Metabolites

A 3 ml cell suspension was sampled rapidly into 9 ml 60% (v/v) cold methanol quenching solutions (<-50°C). The solution was mixed thoroughly and centrifuged for 5 min at -9°C with 5000 ×*g*. Then, the supernatant was separated from the cell pellet. The intracellular metabolites were extracted from the cell pellets using a cold methanol extraction method with a slight modification from Maharjan and Ferenci [14]. Cell pellets were quickly vortexed in the presence of 5 ml of cold (<-50°C) aqueous methanol (60% v/v) and frozen in

liquid nitrogen. The sample was then thawed in an ultrasonic bath for 15 min and centrifuged for 5 min at 5000 ×*g*. Next, the samples were freeze dried at low temperature and stored at −80°C for further analysis. Each extraction was performed in three biological replicates and three technical replicates.

**Sample Derivatization Using Trimethylsilyl (TMS) Method**

A two-step methoximation/silylation derivatization method was used for the samples derivative. First, the dried samples were methoximated with a solution of 10 µl of 4% (w/v) methoxyamine hydrochloride solution in anhydrous pyridine. Then, the mixtures were incubated in 30°C water bath for 90 min followed by silylated with 90 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) at 37°C for another 30 min. After derivatization, a 10 ml of 1 mM of 2-fluorobiphenyl in anhydrous pyridine was injected to the samples as standard. The final incubated mixture was transferred to a GC-MS vial and analyzed by GC-MS.

**Metabolite Analysis Using Gas Chromatography Mass Spectrometry**

Gas chromatography-mass spectrometry analysis was performed on a Shimadzu GCMS-QP2010 Plus. A 0.2 µl of each derivatized sample was injected into 30 m × 0.25 mm i.d. (inner diameter) × 0.25 µm film thin fused-silica capillary column (GC-2010). The injection temperature was set to 250°C, with the septum purge flow rate 3.0 ml $min^{-1}$ and held for 15.50 min. Then, the ion source temperature was adjusted to 230°C. The injection mode was split and the flow control mode was set to a linear velocity of 36.5 cm $sec^{-1}$ and pressure of 57.4 kPa. The column oven temperature was held at 60°C for 1 min, and then increased to 325°C with constant helium gas flow 10 ml $min^{-1}$.

**Data Analysis and Validation Using Multivariate Analysis (MVA)**

Raw GC-MS data were processed using GC-MS Solution software (Shimadzu Japan). Peak identification was carried out using National Institute of Standards and Technology mass spectral library (NIST08s) with the similarities index more than 70%. The metabolomics data were transferred to Microsoft Excel manually. Then, the data were processed in a way that all the identified solvents and foreign substances which were not derived from yeast were removed from the original peak table before normalization. MetaboAnalyst 3.0 server (http://www.metaboanalyst.ca) was used for data integral normalization and statistical analysis [15]. During data filtering, no filter features was set. In the next step, data tables were normalized by two types of normalization. Initially, a row-wise normalization was performed by having the total sum of GC height and then, the column-wise normalization was done by Pareto scaling approach.

One-way Analysis of Variance (ANOVA) was used to statistically validate the values followed by comparison using Fisher's least significant difference (LSD) method with significance levels of $P < 0.05$, $P < 0.01$ and $P < 0.001$. Finally, the normalized and validated data were carried out using principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) of Simca-P+ version 12.0 (Umetrics AB, Ume, Sweden) for group classification and discrimination analysis with Q2 value > 50%. The heat map with hierarchical clustering analysis was performed using MetaboAnalyst 3.0.

**Metabolic Pathway Prediction**

The list of normalized metabolomics data tables containing KEGG/HMBD IDs data was imported into MBrole 2.0 (http://csbg.cnb.csic.es/mbrole2/analysis.php), KEGG Mapper and MetPA in MetaboAnalyst. In MBrole 2.0, all sets of intracellular metabolomics data were annotated individually with KEGG pathway database. The general pathway regarding lipase also known as acylglycerol hydrolases (E.C.3.1.1.3) was searched in KEGG pathway database. A biosynthetic pathway was manually reconstructed by using the outcomes of the metabolic pathway analysis.

**The Effect of Fatty Acids Supplementation on the Thermostable T1 Lipase Expression in Strain SO2**

A single colony of recombinant SO2 was inoculated into YPD and proceeded with 1% (v/v) inoculation into 100 ml YPTG medium until OD600nm ~ 15. Then, the cells were pelleted and resuspended into 100 ml YPT. Commercial fatty acids octanoic acid-C8, decanoic acid-C10, dodecanoic acid-C12, tetradecanoic acid-C14, hexadecanoic acid-C16 and octadecanoic acid-C18 were used for the analysis. A 40 mg of the fatty acids were dissolved with 0.5% methanol and supplemented into the culture every 12 h interval for 3 days. Subsequently, the lipase activity was assayed to determine the effect of fatty acids in the expression yield according to the previously described method.

**RESULTS**

**Intracellular Metabolite Profiling**

The result shows the metabolic contents of *M. guilliermondii* strain wild-type SO and recombinant SO2 extracted with methanol at different time points (0 h and 60 h). In order to assess the different metabolites present in *M. guilliermondii*, the data matrices of 72 samples (8 samples x 3 biological replicates and 3 technical replicates) were subjected to PCA and PLS-DA. The overall multivariate analysis of intracellular metabolite data was collated. Figure 1, describes the PCA score plot of different time point in wild-type SO and recombinant SO2. The separation between

samples was achieved using the first two principal components (PCs) (PC1 versus PC2) with a total variance of 82.38%. PC1 was the highest variance (72.46%) while PC2 was the second greatest variance (9.92%) in subspace perpendicular to PC1. The overall patterns demonstrated a clear separation with $R^2X$ and $Q^2$ (cumulative) value of 0.825 and 0.642, respectively. From the score of PCA, clear separation between samples of different time points was achieved (Figure 1). Wild-type SO at 0 h, showed the highest reproducibility, followed by SO at 60 h, SO2 at 60 h and lastly by SO2 at 0 h. The score plot of PCA analysis showed the separation of the samples, suggesting that the effect of time point was greater than the strain of yeasts. Consequently, the samples were classified in the same time point treatment relative to the strains.

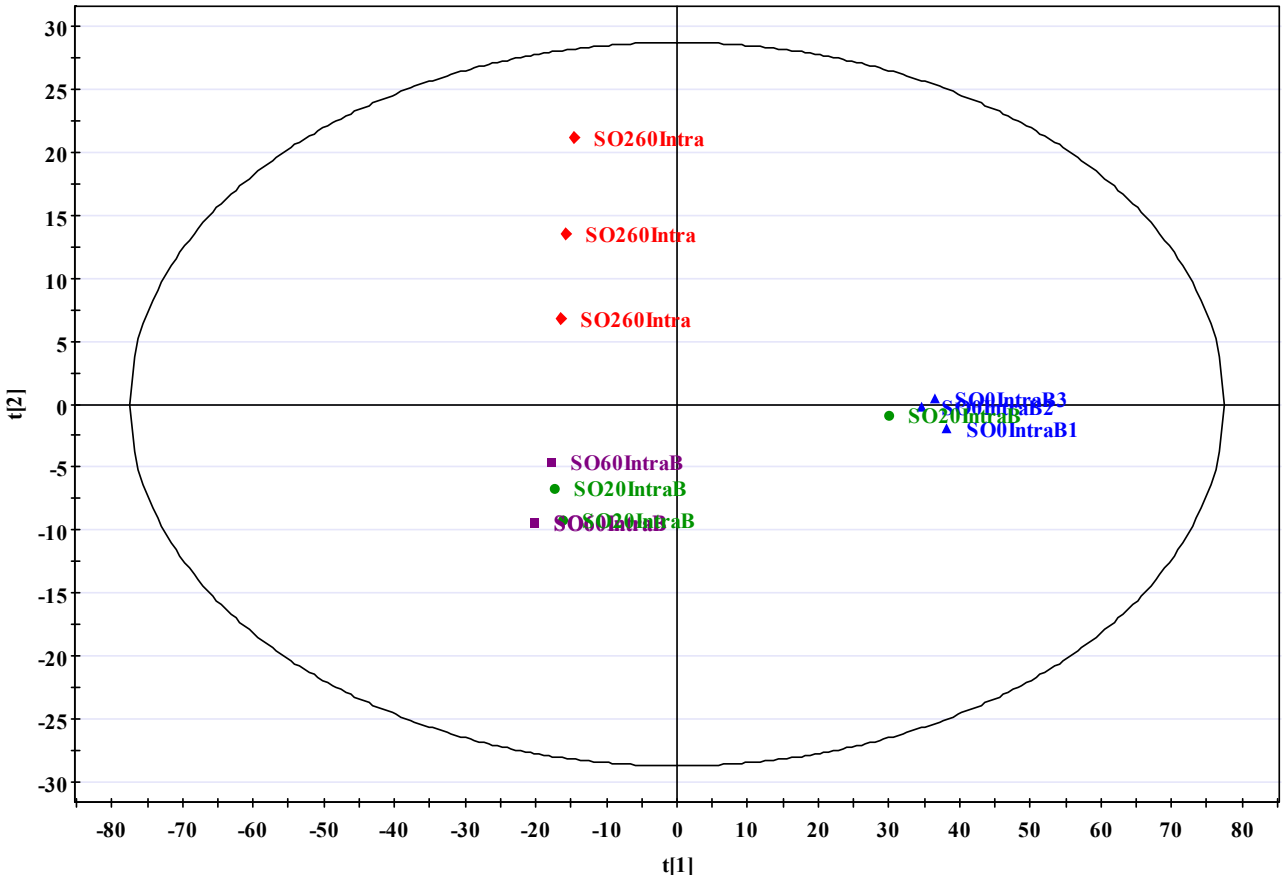


**Figure 1.** Multivariate data analysis of intracellular metabolite data. A score plot of PCA the samples SO and SO2 extracted from intracellular environment of different time points of cultivation. Score plot was generated using the first two PCs (PC1 versus PC2). SO 0 h: wild-type SO at 0 h; SO 60 h: wild-type SO at 60 h; SO2 0 h: recombinant SO2 at 0 h; SO2 60 h: recombinant SO2 at 60 h.

The PCA loading plot shows that PC1 was largely dominated by a bunch of long chain fatty acids such as monounsaturated octadecenoic acid, hexadecenoic acid, diunsaturated octadecadienoic acid, eicosadienoic acid and saturated eicosanoic acid (Figure 2). Meanwhile, PC2 was dominated by saturated fatty acids like octadecanoic acid, hexadecanoic acid and eicosanebioic acid. The distribution pattern of SO at 0 h was mainly influenced by the presence of eicosanebioic acid whereas SO at 60 h was affected largely by the presence of octadecanoic acid and hexadecanoic acid. This occurrence could be due to the action of triglycerides hydrolysis by native lipase in wild-type SO. According to Oslan *et al*., [8], about 0.184 U $mg^{-1}$ of specific activity is observed after the culture was assayed at 30°C. Besides, SO2 at 60 h was influenced by long chain fatty acids such as octadecenoic acid, hexadecenoic acid, octadecadienoic acid, eicosadienoic acid and saturated eicosanoic acid.

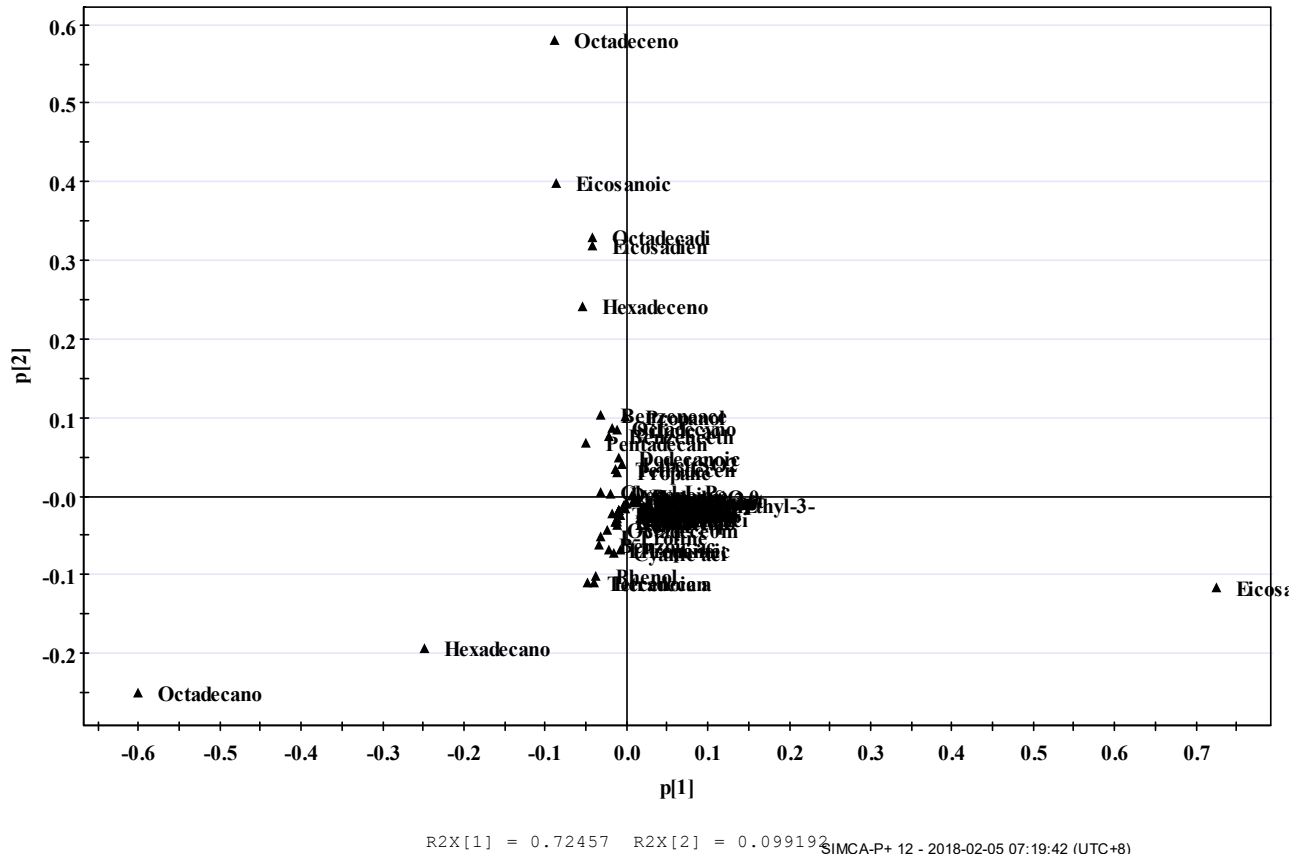


**Figure 2.** A loading scatter plot of PCA analysis of intracellular samples. The plot was generated using the first two PCs (PC1 versus PC2). Each point in the plot of the property represents the metabolites extracted.

As compared to the separation pattern of PCA (Figure 1), the clustering of samples in PLSDA (Figure 2) was similar to PCA. This occurred because the variation within-group was sufficiently lesser than between-group variation. As explained in Worley and Powers [16], the unsupervised nature of the PCA algorithm only revealed group structures when within-group variation was sufficiently lesser than between-group variation. The PLSDA model illustrated in Figure S1a, shows the first two latent variables (LVs) (LV1 versus LV2) to explain the separation between different time point of extraction of intracellular of SO and SO2. A total variance of 82.2% ($R_2X$) was achieved with $R_2Y$ (cumulative) value of 58.8% which exceeding 50%.

Also, Figure S1b shows the loading plots of the metabolites responsible for separation patterns in Figure S1a. The metabolites in red square indicate the metabolites which contained the VIP scores more than 1.0. The distribution pattern of SO at 0 h was mainly influenced by the presence of eicosanebioic acid whereas SO at 60 h was affected largely by the presence of octadecanoic acid and hexadecanoic acid. In addition, Figure S2a, shows the descending order of metabolites with the VIP scores where the higher the scores, the larger the weight of that particular metabolites present in the sample. As shown in Figure S2a, the highest VIP score was observed in eicosanebioic acid (VIP = 3.25), indicating that eicosanebioic acid was the most significant metabolites/variables in the samples. Whereas, Figure S2b, shows the validation of PLSDA model performed by 100 permutation test. The stability and credibility of the model were supported by all the permuted $R^2$ and $Q^2$ values on the left which were lesser than the original point on the right. The $Q_2$-intercept value was negative (-0.313), which meant no over-fitting.

## Extracellular Metabolite Profiling

The result shows the PCA score plot of extracellular samples distribution at different time point in SO and SO2 (Figure 3). Similar to intracellular profiling, the separation between samples was achieved from PC1 versus PC2 with a total variance of 42.40%. PC1 contained the highest variance with 23.53% while PC2 with 18.87%. The $R^2$ and the $Q^2$ obtained were 0.425 and -0.217, respectively. A negative value of $Q^2$ for the plot model constructed explained that the model was obtained from an empirical analysis. Usually, $Q^2$ values increased along with $R^2$, but when noisy variables were added to the model, $Q^2$ values decreased. Consequently, the prediction power of the model was lowered [17].

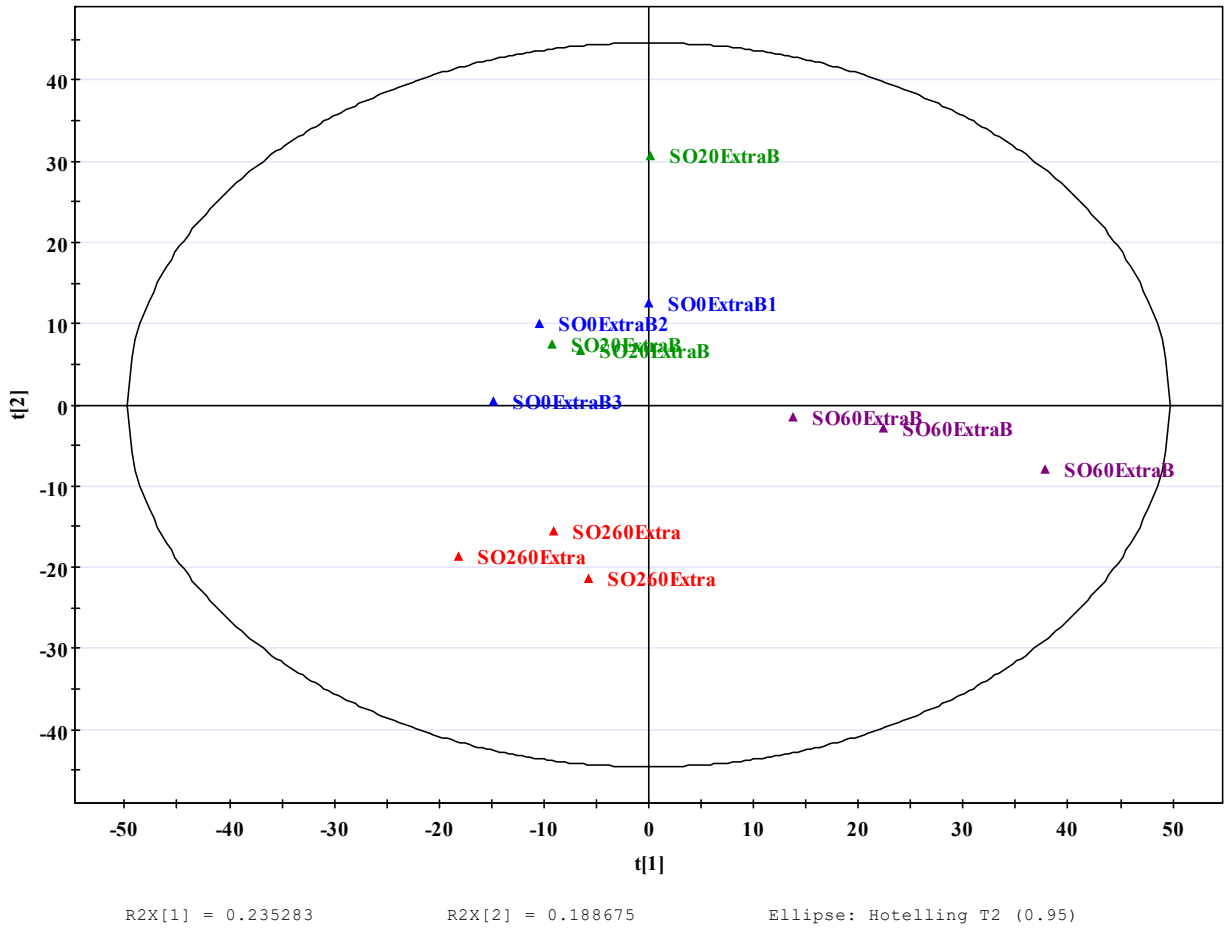


**Figure 3.** Multivariate data analysis of extracellular metabolite data. A score plot of PCA of the samples SO and SO2 extracted from extracellular environment of different time points of cultivation. Score plots of extracellular samples were generated using the first two PCs (PC1 versus PC2). SO 0 h: wild-type SO at 0 h; SO 60 h: wild-type SO at 60 h; SO2 0 h: recombinant SO2 at 0 h; SO2 60 h: recombinant SO2 at 60 h.

The PCA loading plot shows that PC1 was dominated largely by eicosanebioic acid, octadecanoic acid and benzeneacetic acid (Figure 4). PC2 was dominated by propanol and tryptophan. From the PCA score, collected samples at 60 h showed the highest reproducibility as compared to samples at 0 h. The distribution of SO2 at 60 h was mainly influenced by eicosanebioic acid and tryptophan whereas SO at 60 h was affected largely by the present of benzeneacetic acid. Besides, SO at 0 h was influenced by octadecenoic acid and some amino acids (leucine and proline). However, SO at 0 and 60 h showed no clear separation because it shared the metabolites present in SO at 0 h and SO at 60 h which drove the unclear separations.

The PLSDA model demonstrated in Figure S3a, shows the first two latent variables (LV1 versus LV2) to explain the separation between different extraction time point for SO and SO2. A total variance of 41.48% ($R_2X$) was achieved with $R_2Y$ (cumulative) value of 62.3% which exceeded 50%.

When compared to the separation pattern of score plot of PCA (Figure 3), the score plot of PLSDA (Figure S3a), showed even clearer clustering of samples between the extracellular samples of SO at 0 h and SO2 at 0 h. From the score plot of PLSDA, the highest reproducibility was similar to the PCA loading plot pattern (Figure 4). The distribution of SO2 at 60 h was mainly influenced by the presence of eicosanebioic acid and tryptophan whereas SO at 60 h was affected largely by the presence of benzeneacetic acid. Besides, the separation of SO at 0 h clustering was influenced by octadecenoic acid and hexadecanoic acid, while the grouping for SO2 at 0 h was affected by propanol and amino acids (leucine and proline).

Figure S3b, shows the PLSDA loading plots, with the metabolites responsible for the separation patterns in Figure S3a. The interpretation of PLSDA model was further carried out using VIP scores. The metabolites in red square indicates the metabolites which have the VIP scores exceeding 1.0. The PLSDA loading plot shows that LV1 was dominated largely by eicosanebioic acid at right side and benzeneacetic acid at the left side of the plot. LV2 was dominated by propanol, amino acids like leucine and proline, glycylproline

and uric acid. At the other end LV2 was dominated by tryptophan.

Figure S4a, shows the descending order of metabolites with their VIP scores. The higher the VIP scores, the larger the weight of that particular metabolites present in the samples. The highest VIP scores was observed in bezeneacetic acid followed by eicosanebioic acid with the VIP score of 3.15 and 2.75, respectively, indicating that bezeneacetic acid and eicosanebioic acid were the most significant metabolites/variables present in the samples. In addition, Figure S4b shows that the validation of PLSDA model was performed by 100 permutation tests. The model was stable, credible and not over fitted as the permuted $R^2$ and $Q^2$ values were less than the original values and $Q^2$-intercept value is negative (-0.126).

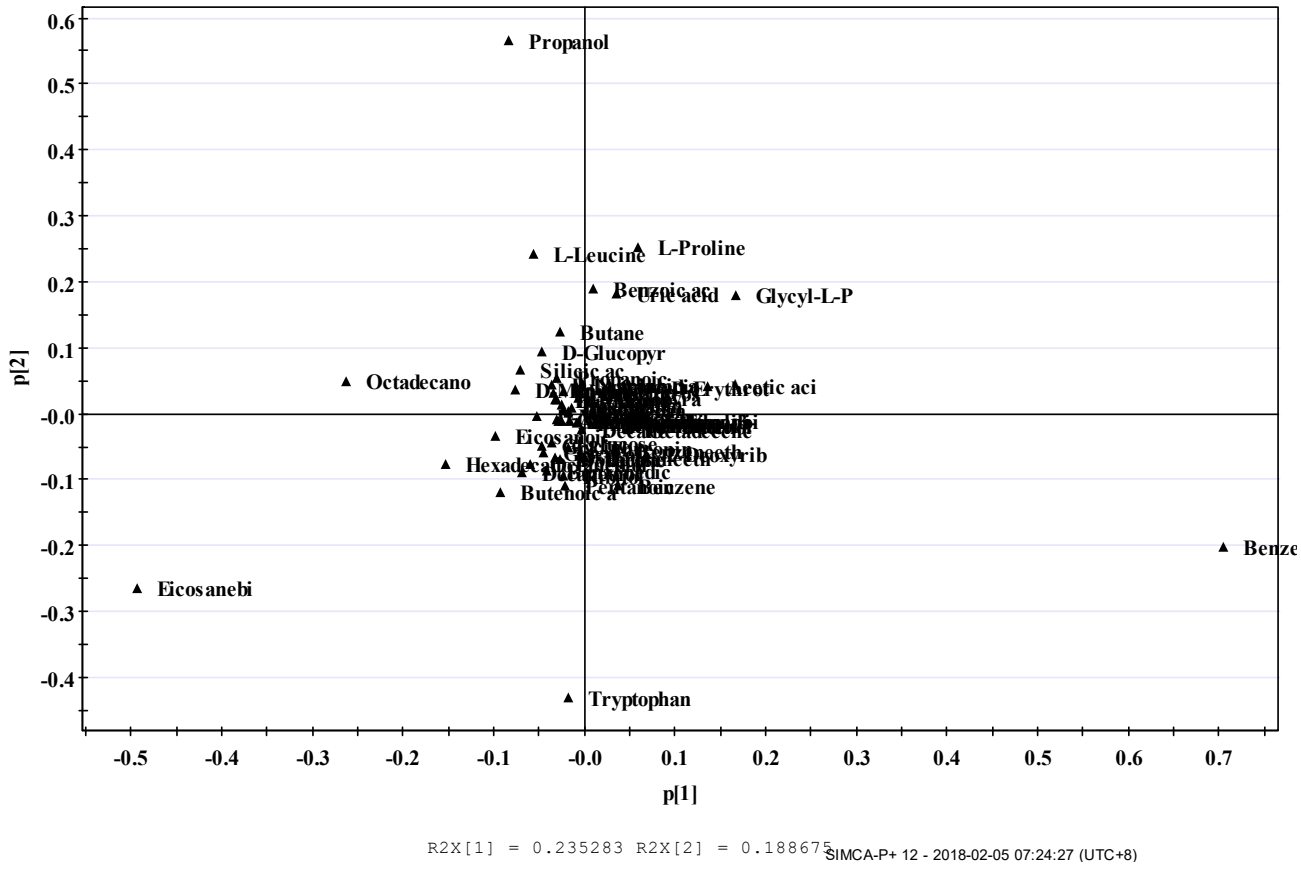


**Figure 4.** A loading scatter plot of PCA analysis of extracellular samples was generated using the first two PCs (PC1 versus PC2). Each point in the plot of the property represents the metabolites extracted.

## Metabolic Pathway Prediction

A pathway was reconstructed manually to include all relevant searches from MBrole 2.0, MetPA, KEGG mapper and Heatmap (Figure S5). The reconstructed biosynthetic pathway is shown in Figure 3. High abundance of unsaturated fatty acids such as hexadecenoic acid, octadecenoic acid, octadecadienoic acid, eicosanoic acid and eicosadienoic acid were present in recombinant SO2 at 60 h cultivation (Figure 5). The fatty acids were up-regulated after 60 h of cultivation even though they were initially low at 0 h.

## The Effect of Fatty Acids on T1 Lipase Expression

As shown in Figure 6, octanoic acid, decanoic acid and hexadecanoic acid were observed to give an inducing effect on the expression of thermostable T1 lipase with minimal methanol as solubilizing agent. Hexadecanoic acid (C16) at the 12 h of incubation gave an increase of 1.45-fold of expressed recombinant protein compared to the control experiment (without fatty acid induction). On the other hand, dodecanoic acid (C12) had shown some inhibitory effect towards T1 lipase expression.

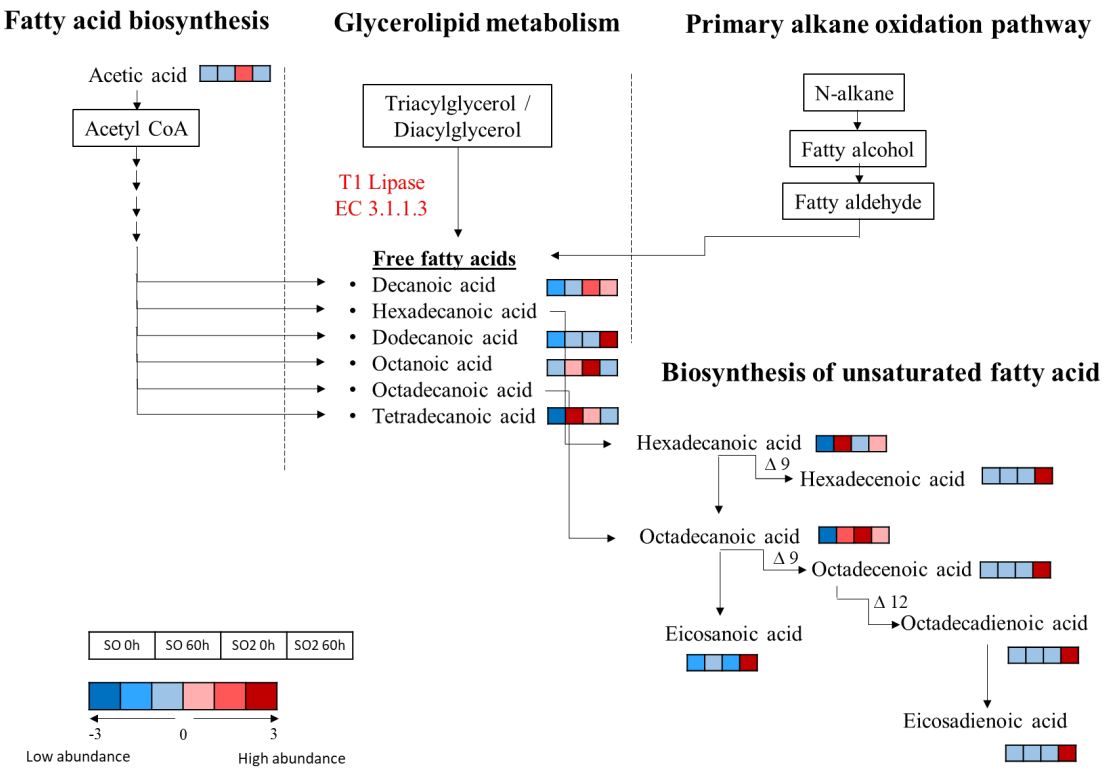


**Figure 5.** A reconstructed pathway displayed with level of fatty acids. The pathway is adapted from the KEGG database. Each column represents the samples from different time point with SO and SO2. (Left to right: SO 0 h: wild-type SO at 0 h; SO 60 h: wild-type SO at 60 h; SO2 0 h: recombinant SO2 at 0 h; SO2 60 h: recombinant SO2 at 60 h).

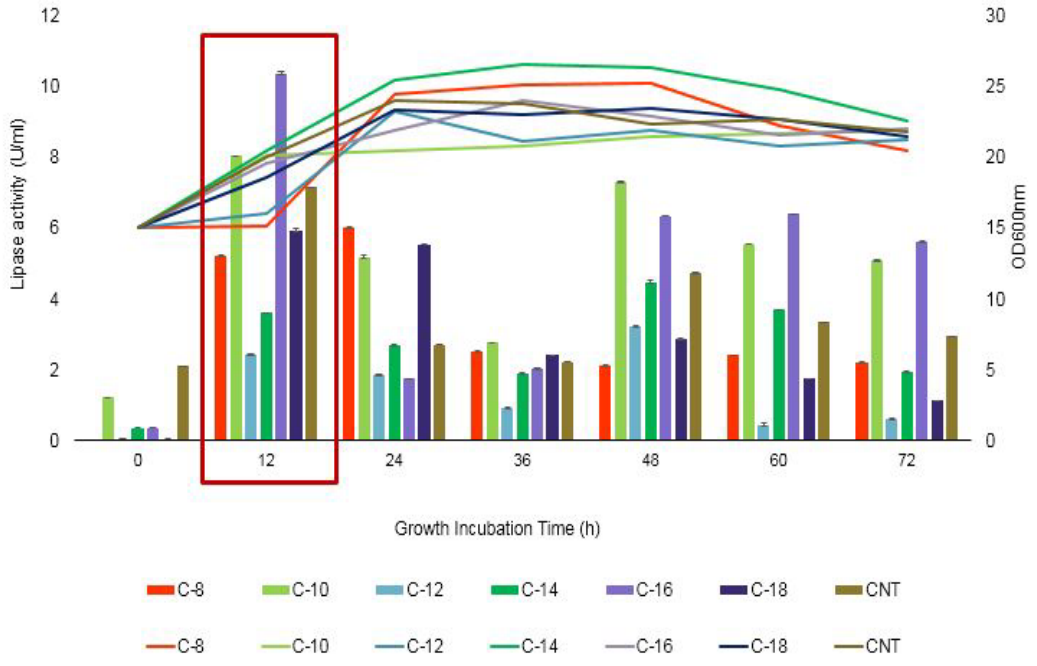


**Figure 6.** The fatty acids supplementation at every 12 h interval, with the highest activity was at the 12 h of incubation and the cell growth was recorded at $OD_{600nm}$. Data means ±standard deviation of three replicates calculated and indicated with error bars.

## DISCUSSION

In the absence of methanol, the optimum T1 lipase expression was found at 60 h in YPT medium (Figure S6). The culture media for both recombinant SO2 and wild-type SO at 60 h in YPT were selected for metabolites extraction. It was expected that the metabolites present at this particular time might contribute to the lipase expression without methanol induction. The strains were initially cultivated in glycerol medium for biomass production and then after, resuspended in the induction medium. According to Gadhi *et al.,* [18], methanol-driven fermentation processes with methylotrophic yeasts typically consists of two phases: biomass growth phase (in glycerol) and a protein production phase (in methanol).

The results from the metabolic profiling of recombinant SO2 showed an abundant amount of fatty acid content as compared to the baseline metabolome reported in *K. phaffii* [19]. Fatty acids could have a significant function in *M. guilliermondii* for its survivability. In previous study, the bacterial T1 lipase was reported to have a successfully expression in *E. coli* intracellularly [13]. In addition, it was reported that the signal peptide did not contribute to the structural gene function of thermostable lipases [20]. However, the T1 lipase gene cloned in strain SO was fused with α-MF signal sequence which functioned to secrete the recombinant protein extracellularly into the medium.

Besides, many studies reported that some amount of recombinant protein fused with α-MF signal sequence failed to be secreted out, thus, remained in the cell. Lin-Cereghino *et al.,* [21] reported that the intracellular lipase activity was detected even though the lipase was fused to α-MF signal peptide. In order to improve the secretion, the mutation was done on the signal sequence.

On the other hand, Rakestraw *et al.,* [22] described vacuolar sorting proteins directing the pro-protein from the Golgi apparatus to the vacuole resulting in misleading of the heterologous proteins, where the protein was detected in the vacuole. These studies imply that there could be a possibility that some T1 lipase might still be present intracellularly prior to its fully secretion. Besides, a few studies worked to improve the efficiency of secretory α-factor such as codon optimization, directed evolution, insertion of spacers and deletion mutagenesis [23].

*M. guilliermondii* has 100% similarity of alcohol oxidase promoter ($P_{AOX1}$) as in methylotrophic yeast like *K. pastoris* [8]. However, the alcohol oxidase present in *M. guilliermondii* ATCC6260 is a long-chain fatty alcohol oxidase (FAO) instead of short chain alcohol oxidase (AOX) that is usually present in methylotrophic yeasts [24]. The substrates of FAO are alcohols with carbon atom length ranging from of 7 to 16 [25] and the best substrate is dodecyl alcohol [26]. This implies that alcohol oxidase promoter $P_{AOX1}$ could be auto-induced by these alcohols, thus would result to the transcription and translation of the recombinant proteins. Cheng *et al.,* [27] reported that *Candida* yeast's long chain fatty alcohol oxidase played an important role in long chain fatty acid metabolism. Nevertheless, the *in vivo* requirements for individual genes with respect to specific substrates are still being elucidated.

Figure 5, shows the presence of fatty acids that could be from the action of T1 lipase which hydrolyzed the stored lipid, triacylglyceride to free fatty acids. Limited information was reported on fatty acid metabolic pathways and production in *K. phaffii*. However, Tomàs-Tomàs-Gamisans *et al.,* [28], reported that the *de novo* biosynthesis of *P. pastoris* also has fatty acid elongation enzymes, which are able to extend $C_{12-14}$ fatty acids and generate very long chain fatty acids (up to $C_{26}$). This report elucidated the presence of long chain fatty acids in the intracellular environment of isolate SO after 60 h of cultivation. Fatty acids have also been reported to serve as the sole carbon source in yeast cells which can induce the expression of recombinant proteins that influences the expansion of the peroxisomal compartment [29].

In this study, it was proven that a significant amount of fatty acids was observed when T1 lipase was optimally expressed after 60 h cultivation. The prompted questions was; why are these fatty acids detected at this particular time and what is the effect on the T1 lipase production is yet to be understood. But, based on this preliminary result, octanoic acid, decanoic acid and hexadecanoic acids have shown their positive effect in supporting the expression of T1 lipase with minimal methanol induction. This result could be used to further determine the alternative inducer for T1 lipase expression in strain SO2 and eradicating the toxicity of methanol induction.

## CONCLUSION

In conclusion, a significant number of fatty acids (long chain fatty acid) were detected when lipase was expressed at 60 h of cultivation. These metabolites, particularly the fatty acids might have contributed to the expression of bacterial T1 lipase without methanol induction, as further analysis with the fatty acids indicated an inducing effect with 1.45-fold of the expressed recombinant T1 lipase compared to the control sample. Hence, the innate fatty acid (C16) metabolite of *M. guilliermondii* strain SO might serve as an alternative inducer, eliminating the toxicity of methanol expressed recombinant proteins that can find various applications in certain foods, cosmetics and pharmaceutical industries.

Further study can be carried out to optimize the identified fatty acids and gene knockout can be performed to validate this postulated alternative inducer.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

## REFERENCES

1. Zhang, A., Sun, H., Wang, P., Han, Y., and Wang, X. (2012) Modern analytical techniques in metabolomics analysis. *Analyst* 137, 293-300.
2. Tyagi1, S., Raghvendra, Usha, S., Taruna, K., and Kavita, M. (2010) Application of metabolomics – A systematic study of the unique chemical fingerprints: An overview. *Int. J. Pharm. Sci. Rev. Res,* 3, 83-86.
3. Cascante, M. and Marin, S. (2008) Metabolomics and fluxomics approaches. *Essays Biochem.* 45 (2008) 67-82.
4. Semreen, M. H., Solima, S. S. M., Saeed, B. Q., Alqarihi, A., Uppuluri, P., and AIbrahim, A. S. (2019) Metabolic profiling of *Candiad auris,* a newly-emerging Multi Drug Resistant *Candida* Species, by GC-MS. *Molecules* 24(3), 399.
5. Macauley-Patrick, S., Fazenda, M. L., Mcneil, B. and Harvey, L. M. (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 22, 249–270.
6. Sibirny, A. A., and Boretsky, Y. R. (2009) *Pichia guilliermondii*. In *T. Satyanarayana, G. Kunze (Eds.), Yeast Biotechnology:* Diversity and Applications: Springer Science + Business Media B.V, New Delhi, India, (pp. 113-134).
7. Oslan, S.N., Salleh, A.B., Rahman, R.N.Z.R.A., Basri, M. and Leow, T.C. (2012) Locally isolated yeasts from Malaysia: Identification, phylogenetic study and characterization. *Acta Biochim. Pol.* 59, 225-229.
8. Oslan, S. N., Salleh, A. B., Rahman, R. N. Z. R. A., Leow, T. C., Sukamat, H., and Basri, M. (2015) A newly isolated yeast as an expression host for recombinant Lipase. *Cell. Mol. Biol. Lett.* 20(2), 279–293.
9. Abu, L. M., Nooh, H.M., Oslan, S.N., and Salleh, A.B. (2017) Optimization of physical conditions for the production of thermostable T1 lipase in *Pichia guilliermondii* strain SO using Response Surface Methodology. *BMC Biotechnol.* 17, 78.
10. Vogl, T., Sturmberger, L., Fauland, P.C., Hyden, P., Fischer, J.E., Schmid, C., Thallinger, G.G., Geier, M., and Glieder, A. (2018) Methanol independent induction in *Pichia pastoris* by simple derepressed overexpression of single transcription factors. *Biotechnol. Bioeng.* 115*,* 1037–1050.
11. Oslan, S. N., Salleh, A. B., Rahman, R. N. Z. R. A., Leow, T. C., and Basri, M. (2014) *Pichia Pastoris* as a host to overexpress the thermostable T1 lipase from *Geobacillus zalihae*. *GSTF Int. J. Bio. Biotech.* 3(1), 7.
12. Kwon, D.Y. and Rhee, J.S. (1986) A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *J. Am. Oil Chem. Soc*. 63, 89-92.
13. Leow, T.C., Rahman, R.N.Z.R.A., Basri, M., and Salleh, A.B. (2007) A thermoalkaliphilic lipase of *Geobacillus* sp. T1. Extremophiles. 11, 527-535.
14. Maharjan, R.P. and Ferenci, T. (2003) Global metabolite analysis: the influence of extraction methodology on metabolome profiles of *Escherichia coli*. *Anal. Biochem.* 313, 145–154.
15. Xia, J., Sinelnikov, I. V., Han, B., and Wishart, D. S. (2015) MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Res.* 1, W251–W257.
16. Worley, B. (2013) Powers, R, Multivariate analysis in metabolomics. *Curr. Metabolomics.* 1, 92–107.
17. Consonni, V., Ballabio, D., and Todeschini, R. (2009) Comments on the definition of the $Q^2$ parameter for QSAR validation. *J. Chem. Inf. Model.* 49, 1669-1678.
18. Gandhi, S., Salleh, A.B., Rahman, R.N.Z.R.A., Leow, T.C., and Oslan, S.N. (2015) Expression and characterization of *Geobacillus stearothermophilus* SR74 recombinant α-amylase in *Pichia pastoris*. *Bio. Med. Res. Int.* 9, 18.
19. Tredwell, G.D., Edwards-Jones, B., Leak, D.J., and Bundy, J.G. (2011) The development of metabolomic sampling procedures for *Pichia pastoris*, and baseline metabolome data. *PLOS ONE.* 6, e16286.
20. Leow, T. C. (2005) Molecular studies, characterization and structure elucidation of a thermostable lipase from *Geobacillus* sp. PhD Thesis, Universiti Putra Malaysia, Serdang, Selangor.
21. Lin-Cereghino, G.P., Stark, C M., Kim, D., Chang, J.W.J., Shaheen, N., Poerwanto, H., Agari, K., Moua, P., Low, L.K., Tran, N., Huang, A.D., Nattestad, M., Oshiro, K.T., Chavan, A., Tsai, J.W., and Lin-Cereghino, J. (2013) The effect of α-mating factor secretion signal mutations on recombinant protein expression in *Pichia pastoris*. *Gene* 519, 311–317.
22. Rakestraw, J. A., Sazinsky, S. L., Piatesi, A., Antipov, E., and Wittrup, K. D. (2009) Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 103, 1192–1201.
23. Ahmad, M., Hirz, M., Pichler, H., and Schwab, H. (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl. Microbiol. Biotechnol.* 98, 5301–5317.
24. Crisol, G.J. (2017) Identification and classification of alcohol oxidases from yeast using bioinformatics approach. Bachelor of Science (Hons) Biochemistry Thesis, 2017, Universiti Putra Malaysia.
25. Pickl, M., Fuchs, M., Glueck, S. M., and Faber, K. (2015) The substrate tolerance of alcohol oxidases. *Appl. Microbiol. Biotechnol.* 99, 6617–6642.
26. Dickinson, F. M. and Wadforth, C. (1992) Purification and some properties of alcohol oxidase from alkane-grown *Candida tropicalis*. *Biochem. J.* 282, 325-331.
27. Cheng, Q., Sanglard, D., Vanhanen, S., Liu, H.T., Bombelli, P., Smith, A., and Slabas, A.R. (2005) *Candida* yeast long chain fatty alcohol oxidase is a c-type haemoprotein and plays an important role in long chain fatty acid *metabolism. Biochim. Biophys. Acta.* 1735, 192-203.
28. Tomàs-Gamisans, M., Ferrer, P., and Albiol, J. (2016) Integration and validation of the genome-scale metabolic models of *Pichia pastoris*: A comprehensive update of protein glycosylation pathways, lipid and energy metabolism. *PLOS ONE* 11, e0148031.
29. Gurvitz, A. and Rottensteiner, H. (2006) The biochemistry of oleate induction: Transcriptional upregulation and peroxisome proliferation. *Biochim. Biophys. Acta, Mol. Cell Res.* 1763 (12), 1392-1402.