Parameters for Novel Production of Fruity Floral Fragrance Ester (Geranyl Butyrate) by Locally Isolated Lipase *Geobacillus thermodenitrificans* nr68 (LGT)

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**Abstract.** Geranyl butyrate has been synthesized successfully using our locally isolated lipase *Geobacillus thermodenitrificans* nr68 (LGT) as the fragrance ester with aim to be used in a nanotechnology fragrance application. We have used and modified few parameters from the previous research and then, continued with optimization of the synthesis by looking into degree of esterification and water content in the system. Butyric acid (C4), stearic acid (C18: 0), caprylic acid (C8), linolenic acid (C18: 3), myristic acid (C14), linoleic acid (C18: 2) and oleic acid (C18: 1) were used in the substrate selection. The yield of geranyl butyrate before the optimization was 31.68±0.01%. The optimum parameters for the synthesis of geranyl butyrate were recorded as temperature of 65°C, shaking rate at 200 rpm, 5.0 ml of geraniol and 0.40 ml of butyric acid and 4.0 ml of n-butanol and 0.40 ml of oleic acid. After the optimization, geranyl butyrate synthesis was increased by 297% as to compare with the value before the parameters were optimized. We also have significantly reduced water content as a byproduct of the esterification and managed to run the system a success. The ability thermotolerant lipase from *Geobacillus thermodenitrificans* (LGT) in this synthesis is novel to Malaysian fragrance industry.

1. **Introduction**
Fragrance and aromatic compounds are used widely in most food industries, dairy products and detergent. The extraction of both compounds from plants has been recognized to be involving high cost as well as the source is limited to certain kinds of plants. Thus, the compounds are now widely produced by microbial sources through a process called biotransformation [1] in order to overcome the above two issues. Biotransformation activities for the production of these aromatic compounds have started last decade [2]. The process is supported very well by the use of extracellular lipases and the enzyme high affinity in organic solvent. Thermostable enzyme with high specification is now chosen at the industrial level because it is very stable at elevated temperatures and is easy to handle when it comes to involving the organic solvent. Locally isolated species is focused as the potential enzyme producers as to create a novel production and with better control over the large scale production [3]. Lipase from *Geobacillus thermodenitrificans* (LGT) has been used to study the substrate specification for the purpose of producing potential fragrance; geranyl butyrate and butyl oleate.

2. **Materials and Method**

2.1 **Chemicals**
Butyric acid (C4), stearic acid (C18: 0), caprylic acid (C8), linolenic acid (C18: 3), myristic acid (C14), linoleic acid (C18: 2) and oleic acid (C18: 1) are products of Sigma-Aldrich. Other chemicals were terpene alcohol (Sigma), molecular sieve (Merck), skimmed-milk (Island Farms), copper (II)
acetate (Sigma-Aldrich), pyridine acetate (Sigma-Aldrich), benzene (Sigma), heptane (Merck),
tetrahydrofuran (Sigma-Aldrich), ethyl acetate (99.8%, Sigma-Aldrich), isooctane (Sigma), toluene
(anhydrous, 99.8%, Sigma-Aldrich), hexane (Sigma) and heptane (Sigma).

2.2 Preparation of freeze-dried lipase enzyme
Method by [1] has been adapted. The 48h Geobacillus thermodenitrificans fermentation broth was
centrifuged using High Speed Centrifuge Refrigerated 6500 model; Kubota. Crude lipase enzyme
activity of 16.68U/ml was concentrated by removing up to 80% of the volume of water from the
extract using Viva flow machine (Cole Parmer, MASTERFLEX C/S). Concentrated lipase activity
was recorded as 19.82-22.20 U/ml. Then, a total of 20% fat-free skimmed milk was added into the
concentrated enzyme followed by freeze-drying process conducted in 1500-2000 millitorr vacuum;
condensing at-50ºC using a Bench Top 2K (VirTis). Collected lipase powder activity was recorded as
30.50 U/g. Figure 1 shows the freeze-dried LGT.

![Figure 1: The freeze-dried LGT](image)

2.3 Esterification and degree of esterification.
10.0% (v/v) different kind of fatty acids, 10.0% (v/v) terpene alcohol (geraniol), 10.0% (w/v) LGT,
10.0% (w/v) molecular sieve and 80.0% (v/v) organic solvent were used at 200rpm and 65ºC [4].The
degree of esterification was determined every 6 h for 72h and the test was conducted in triplicate.
Modification of copper soap colorimetry method by Saisuburamanian et al. [5] was applied. Copper
acetate-pyridine reagents contained 5% copper acetate [Cu(AC)] was filtered and then adjusted to a
pH of 6.1 using pyridine. It was to determine the amount of fatty acids left behind after the reaction.
0.40 ml of reaction mixture was transferred to a test tube, followed by 4.60 ml of benzene and before it
was vortex to dissolve the sample. Cupric solution of 1.0ml pyridine acetate was added and vortex (2
min). The optical density at 715 nm (Spectrophotometer Spectronic UNICAM 10uV Genesys) was
recorded and expressed through the exchange of free fatty acids in the µmol using 0-1000 µmol
standard curve containing pure oleic acid. The pure oleic acid was prepared in 4.0 ml of heptane,
followed by the addition of 100 mL copper acetate-pyridine reagent. The optical density at 715 nm
was plotted. The acid concentration was determined based on the standard curve plotted. The degree
of esterification was determined based on the reduction of the concentration of the fatty acids
converted into esters. The degree of esterification is shown in Equation 1.

$$\frac{K_A - K_B \times 100\%}{K_A}$$

(1)

Where;
$K_A =$ concentration of fatty acid in the initial
reaction, $K_B =$ concentration of fatty acids in the
reaction

2.4 Determination of water content in the system
The water produced by the esterification was determined by using Caulometer Titramate (684KF,
Metrohm, Switzerland). The mixture was centrifuged at 10,000 rpm for 5 minutes and 5.0ml of the
clear layer was injected to determine the water content. It was compared with the control and
expressed in unit percentage. The test was carried out thrice. Control was the esterification mixture
without the addition of LGT.
2.5 Substrate for terpene ester synthesis

The substrate was geraniol (~99-95% GC grade, Fluka, Chemika). Fatty acids were used to refine the formation of terpene ester. Methanol was used as organic solvents. All tests were conducted in triplicate [1]. Geranyl butyrate synthesis before optimization process has consisted of 0.50g LGT, 0.50ml geraniol, 0.50ml butyric acid, 4.00ml heptane and 0.50g molecular sieve (0.3 nm) incubated at 65ºC (200rpm). The degree of esterification and water content was determined every 6 h (72 h). All tests were conducted in triplicate. We have recorded the optimum time as 42 h. For the effect of temperature and shaking rates temperature of 55°C-75ºC were used and shaking rates of 0, 50, 100, 150, 200 and 250 rpm [6]. The degree of esterification and water content was determined at 42 h.

The organic solvents tested were benzene, tetrahydrofuran, ethyl acetate, isooctane, toluene, hexane and heptane. This was followed by the weight of molecular sieve (0.25g, 0.50g, 0.75g, 1.00g and 1.25g). The choice of solvents and weight of molecular sieve was done according to Ibrahim et al. [7]. The optimum condition and synthesis of geranyl butyrate under optimum condition was 4.00ml of heptane, 0.25g of molecular sieve, 0.50g of LGT, 0.50ml of geraniol and 0.5ml of butyric acid at 200 rpm and 70ºC [1].

3. Result and Discussions

The study of terpene ester synthesis was conducted using freeze-dried LGT. The skimmed milk has not interrupted the esterification because it has a fat-free response to the system. Terpene ester formation from all studies was confirmed by thin layer chromatography (TLC). Figure 2 shows geranyl butyrate obtained after the synthesis where A is geraniol, B is butyric acid, C is the product (the geranyl butyrate) and D is the reaction mixture for the synthesis of geranyl butyrate after 72h using freeze-dried LGT.

Figure 3 indicates degree of esterification of geraniol with 7 types of fatty acids, the maximum degree of esterification of 35.33 ± 0.48% was recorded with butyric acid. The esterification of geraniol with linoleic acid (C18:2), stearic acid (C18:0) and linolenic acid (C18:3) resulted lower values; 20.88±0.22%, 13.92±0.28% and 4.41±0.55, respectively. According to Maha et al. [8] and Abbas and Comeau [9], lipase activity increases with the increasing of fatty acid chain length and the number of linear carbon atoms to the related acid. However, it is not significant with caprylic acid (C8), myristic acid (C14) and oleic acid (C18:1); there was no esterification happen with geraniol. This condition is believed to be associated with the presence of high quantity of water in the reaction system (0.50-0.93%). Therefore, geraniol may have shown low affinity towards the caprylic acid (C8), myristic acid (C14) and oleic acid (C18:1). Catalysis in organic solvents was significant with only 1-2% water content. Using this quantity or less, certain enzymes may switch the interaction into esterification via hydrolysis which reduces the yield. This condition may have happened to particular substrates with LGT. The study of geranyl butyrate synthesis before the optimization process is illustrated in Figure.4. Heptane was used based on the non-polar hydrophobic characteristic. Heptane has a Log P value of 4.0 and methanol with -0.76. Since a solvent with a high value of Log P seeks to reduce the water content and improve the degree of esterification (through reaction reversible obstruction of ester hydrolysis) better, the hexane was chosen. During the first 6h, the degree of esterification was recorded as 23.87±0.77%. From 12 to 36 h, the values were recorded as consistent (28.67±0.57%-

![Figure 2: Thin layer chromatography (TLC) shows the separation of the components resulting from the synthesis of geranyl butyrate by LGT.](image-url)
The highest degree of esterification was recorded at 42 h with 31.68±0.09%. The esterification began to decline at the 72h with water content recorded as 0.833±0.043%. Synthesis declination can be attributed to an increment in the water content of the system. The first product in ester synthesis by most lipase is water and water activities are a standard use for detecting the enzyme hydration in different solvents; therefore, this situation is suggested to serve for the integrity of the enzyme to allow catalysis running significantly. The geranyl butyrate production has been optimized improve the degree of esterification.

The parameters such as temperature, shaking rates, types of organic solvent, amount of molecular sieve, and ratio of geraniol to butyric acid and LGT concentrations were optimized to enhance the formation of ester. Then, the geranyl butyrate production was carried out under optimum conditions and was compared with the results prior to optimization. LGT has demonstrated a significant effect of temperature on its synthesis of geranyl butyrate. Most microbial lipases used under high temperature in biotechnological applications of detergents, textile industry and the production of surfactant in the presence of organic solvents were mostly reported as a significant success. Figure 5 shows the effect of temperature on the esterification, which is projecting significant values of 38.73±0.784% to 43.89±1.76%. The highest yield of geranyl butyrate was recorded at 70°C with water content of 0.84±0.04%. This result is on surprising since LGT has been isolated from a hyperthermolerant species [1] indicating its excellent protein stability at both high temperature as well as in most organic solvents [10]. At 65°C, the yield was recorded as 43.65±1.036% with water content of 0.79±0.01%. Basically, the yield at 55-75°C didn’t reflect big difference. The water content in the system was quite high suggesting as the effect of high temperatures; therefore, we believed that the synthesis of geranyl butyrate has might be impaired. We strongly believed that without these water effects, the yield will be higher. In most non-aqueous enzymatic system, the water should be controlled to be as low as 0.01% [11], and increment of water content in even very small quantities shall give a huge change to the activity of enzyme catalysis. The enzyme is inactive in the system without water, but, catalytic degrees will increase with the degree of hydration [12]. Thus, the water accumulated in the system of LGT at those temperatures is likely to have a major effect on the yield of geranyl butyrate.

The effect of temperature on the protein enzyme will become more apparent with the presence of high water content. Denaturation effect on the enzyme was found to occur in an environment of water and high temperatures. Studies by Negishi et al. [13] have shown that lipase PL from Alcaligenes sp. has a maximum reaction at 70°C with water content at 0.5% (w/w) of the weight of the enzyme. According to this researcher, when the water content was recorded as 0.1% (w/w) over the lipase PL’s weight, the enzyme’s maximum temperature has rose to 85°C. This situation significantly showed that denaturation process caused by temperature is lower when the water content is low. Most commonly, various types of chemical changes, mono-molecular clotting and non-reversible aggregation take place very quickly at a high temperature and enzyme becomes inactive in lot more situation resulting in failure in synthesis. Since most of these processes involve water and therefore, is not happening in an environment without water the use of organic solvent has been a standard choice to run esterification. The protein structure and the resolution of lipases have been confirmed to be better in organic solvents rather than in aqueous solution. Thus, the unfolding of the enzyme is not easily occurring; consequently, increase the thermostability of the enzyme. The binding force between lipase and water molecules is high featuring to more susceptibility of most enzymes towards deactivation at high temperatures. The effect of shaking rates to the esterification by LGT is illustrated in Figure 6. Maximum yield of the geranyl butyrate was collected at 200 rpm (49.0±0.1%). The water content was low. System containing hydrophobic and hydrophilic substrates is homogenized by the shaking progress. While the rate and duration of the reaction by enzyme is influenced by a variety of factors related to the substrate and enzyme, the process of mixing the substrate and enzyme is important to spread the heat and result in a reaction environment. Unfortunately shaking rate at 250 rpm greatly affects the esterification with yield of only 12.03±0.1% due to potential of enzyme deactivation or side chain demoliished by the activities. Nik Raikhani[1] has stated about ‘excess levels of movement denature the enzyme’. This occurs due to the effect of hacks that are produced during the shaking or stirring and entrainment of air into the medium on the surface of the air and liquid [1].

The enzyme particles will form small fragments in excess of string, then, the water molecules cannot be maintained at the enzyme and thus water will accumulate in the reaction system and interfere with the esterification [14]. Conversely, at a low shaking rate (<100 rpm), aggregation of
enzyme-substrate-water applied the mass transfer which makes it difficult for the esterification reaction goes well. Water had a major impact on the characteristics highlighted by lipase. The effect will change the hydration of enzyme hence indirectly alter the nature of the reaction medium and/or enzyme support system [1]. The effects of organic solvent on the degree of esterification of geranyl butyrate was studied using organic solvent having Log P as follow; heptane (Log P=4.0), hexane (Log P=3.5), isooctane (Log P=4.5), tetrahydrofuran (Log P=0.45), toluene (Log P=2.5), benzene (Log P=2.0) and ethyl acetate (Log P=3.51). The organic solvent that has a value of Log P has high hydrophobic effect and can usually provide an activity or good stability and reduces the deactivation of enzyme. To identify the effects of organic solvent on this synthetic ester, the parameters of the organic solvent itself should be identified carefully. Log P logarithm coefficient is defined as the separation of a component in a dual-phase system octanol-water [15].

Figure 7 shows the effect of organic solvents on the esterification of geranyl butyrate by LGT. Based on Figure 7, the maximum yield of esterification was recorded with heptane of 48.05±0.3% and water content of 0.028±0.01%. Benzene performed the second highest yield (40.23 ± 0.21%) even theoretically solvent with low Log P value (Log P=2.0) usually doesn’t work well for esterification. The water content was 0.149 ± 0.01%. Instead, hexane that has higher Log P value (Log P=3.5) gave a lower yield of geranyl butyrate (20.96±0.10%) even the water content recorded in the system was very low (0.045±0.01%). These results are believed not to be subjected to the h of study but were caused by the characteristics of organic solvents used for each system. Above all, the isooctane with the highest value of Log P (Log P=4.5) didn’t produced enough yield of at least what was promoted by the toluene (Log P=2.5). This situation illustrates that Log P is not a major factor in influencing the synthesis of geranyl butyrate by LGT. Molecular sieves are molecules used to absorb water in inorganic or organic systems. Figure 8 shows the effect of molecular sieve on the yield of geranyl butyrate by LGT. The addition of more than 0.25 g molecular sieve has improved the degree of esterification of about 55%-60%, with moderate water content. However, the yield in the system without the molecular sieve was very not significant (4.29±0.367%). The maximum yield was recorded with 0.25g molecular sieve (91.56±0.1%) with water content of 0.412±0.01%. These results indicated that the molecular sieve’s function is only effective when is used in suitable amount. The reaction medium without or with the use of molecular sieve will cause an increment of water content that would interfere with the esterification system (Figure 8).

Figure 3: Esterification between geraniol with various types of fatty acids. Values are expressed as the mean of triplicate readings.

**Reaction condition:**
0.50ml geraniol, 0.50ml various fatty acids, 4.00ml of methanol, 0.50g molecular sieve & 0.50g LGT
200 rpm, 65°C, 72h

Figure 4: Profile of geranyl butyrate synthesis by LGT (before optimization).

**Reaction condition:**
0.50ml geraniol, 0.50ml butyric acid, 4.00ml heptane, 0.50g molecular sieve & 0.50g LGT
200 rpm, 65°C, 72h, volume of analysis: 0.40 ml
Figure 5: Effect of temperatures on the synthesis of geranyl butyrate by LGT.
**Reaction condition:**
0.50ml geraniol, 0.50ml butyric acid, 4.00ml methanol, 0.50g molecular sieve & 0.50g LGT
200 rpm, 55-75°C, 42h, volume of analysis: 0.40 ml

Figure 6: The effect of shaking rates to the establishment geranyl butyrate by LGT.
**Reaction condition:**
0.50ml geraniol, 0.50ml butyric acid, 4.00ml methanol
0.50g molecular sieve & 0.50g LGT
0, 50-200 rpm, 70°C, 42h, volume of analysis: 0.40 ml

Figure 7: The effects of organic solvent on the esterification of geranyl butyrate by LGT.
**Reaction condition:**
0.50ml geraniol, 0.50ml butyric acid, 4.00ml heptane, hexane, isooctane, tetrahydrofuran, toluene, benzene & ethyl acetate, 0.50g molecular sieve & 0.50g LGT
200 rpm, 70°C, 42 h, volume of analysis: 0.40 ml

Figure 8: The effects of molecular sieve on the esterification of geranyl butyrate by LGT.
**Reaction condition:**
0.50ml geraniol, 0.50ml butyric acid, 4.00ml heptane, 0, 0.25, 0.50, 0.75, 1.00 and 1.25g molecular sieve & 0.50g LGT
200 rpm, 70°C, 42 h, volume of analysis: 0.40 ml
Figure 9: Profile of geranyl butyrate synthesis using optimized parameters catalyzed by freeze-dried LGT in 72 h.

**Reaction condition:**
0.50ml geraniol, 0.50ml butyric acid, 4.00ml heptane, 0.25g molecular sieve & 0.50g LGT
200 rpm, 70°C, 72 h, volume of analysis: 0.40 ml

Table 1 listed details of the system of LGT catalyzation before and after optimization. The yield of geranyl butyrate increased 297% after optimization, a great achievement.

**Table 1**: Parameters before and after optimization of geranyl butyrate synthesis catalysed by LGT

| Parameters                     | Before-optimization | Optimized Condition |
|--------------------------------|---------------------|---------------------|
| 1. Solvent                     | Heptane             | Heptane             |
| 2. Molecular sieve             | 0.50 g              | 0.25 g              |
| 3. Shaking rate                | 200 rpm             | 200 rpm             |
| 4. Temperature                 | 65°C                | 70°C                |
| 5. H of optimum production     | 18 h starting 42 to 60 h | 38 h starting 24 to 60 h |
| 6. Yield                       | 31.68±0.01%         | 125.79±0.03% (297% better yield) |
| 7. Water content               | 0.39±0.01%          | 0.196±0.01%         |

4. Conclusion
By using the optimized parameters of 4.00ml heptane, 0.25g molecular sieve, 0.5g LGT, 0.50ml geraniol and 0.50ml butyric acid, temperature at 70°C and the shaking rate at 200 rpm, we managed to increase the production of geranyl butyrate by 297%, a very excellent result. The ability of thermotolerant lipase from *Geobacillus thermodenitrificans* (LGT) in this synthesis is novel to Malaysian fragrance industry and we are significantly already extended this research to another field, the nanotechnology application using our locally synthesis geranyl butyrate in fragrance industry.

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