Optimization of Culture Conditions on the Proliferation of *Aspergillus Terreus* N-GL1 Strain Isolated from *Curcuma Longa* L. by Design-Expert 6.0.6 and BC Pharsoft Software

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**Abstract.** *Curcuma longa* L. is widely used as spices and medicines. Leaves, stems and rhizomes of the plant contain essential oils and provide a useful habitat for different endophytic fungi groups. Strain of *Aspergillus terreus* was isolated from *Curcuma longa* L. and was determined to be capable of producing secondary metabolites against bacteria by disc diffusion method and optimization of culture conditions by Design-Expert 6.0.6 and BC Pharsoft software. This research identified the culture conditions for *A. terreus* N-GL1 strain produced secondary metabolites against *Staphylococcus aureus* and MRSA (Methicillin resistant *Staphylococcus aureus*) good: appropriate carbon source was saccharose 1% or molasses 2.2%; suitable nitrogen sources are potatoes 10% at pH 7; initial amount of yeast cells was 10\(^4\)CFU/ml; incubated at room temperature for seven day. This study was found out as suitable conditions for *A. terreus* N-GL1 strain produced the secondary metabolites against *S. aureus* and MRSA.

1. Introduction
Endophyte that grow in the deep tissues of the plant, but usually do not cause disease the host plant. Endophytic fungi close relationship with the host, they use the nutrients in the plants to survive, bringing many benefits to plants as generating metabolic products have biological activity as the growth hormone, antibiotics have the ability to protect plants from disease-causing microorganisms. The secretions from the endophyte has antibacterial, antifungal and antioxidant, etc. [7]

Some of the fungi are as *Aspergillus, Penicillium, Fusarium* have produced of many metabolites for biological effects such as antibacterial, antiviral, anticancer, etc. In which *A. terreus* for some compounds antibiotics such as terremid A, terremid B, terrein. *A. terreus* also produces enzymes such as invertase and β-glucosidase when grown in submersible fermentation containing barley flour black as carbon source; production of β-xylanase; cis-aconitic dehydroxylase (CAD) - an important enzyme which produced of itaconic acid, beta-glucosidase, etc. [9]

In this study, *A. terreus* N-GL1 were isolated from the leaf of *Curcuma longa* L. (Zingiberaceae family) has identified capable of produced secondary metabolites against *S. aureus* and MRSA. Research on the impact of environmental conditions on endogenous fungi capable of producing highly active metabolites is a new research direction and potential for future application. *A. terreus* N-GL1 were isolated from the leaf of *Curcuma longa* L. (Zingiberaceae family) has been identified capable of produced secondary
metabolites against \textit{S. aureus} and MRSA. Research on the impact of environmental conditions on endogenous fungi capable of producing highly active metabolites by disc diffusion method and optimization of culture conditions by Design-Expert 6.0.6 and BC Pharsoft software is a new research direction and potential for future application.

2. Materials and Methods

2.1. Materials

N-GL1 strain was isolated from \textit{Curcuma longa} L. (Zingiberaceae family) from the medicinal garden of University of Medicine-Pharmacy HCM City. Microorganisms used for microbiological testing were obtained from ATCC: \textit{Escherichia coli ATCC 25922}, \textit{Staphylococcus aureus ATCC 29213}, \textit{Streptococcus faecalis ATCC 29212}, MRSA ATCC 43300, \textit{Pseudomonas aeruginosa ATCC 27853}, \textit{Candida albicans ATCC 10231}.

2.2. Methods

2.2.1. Identification of N-GL1 strain

The fungal isolates were identified on the basis of colony characteristics (colony growth, colour and production of exudate) and sporulating structures (conidiogenous cells, vesicle, conidial head and conidia). [4]

2.2.2. The effect of different pH media on the production of antibacterial substance of N-GL1

Surveying the effect of pH media on the production of antibacterial substance by N-GL1 on the PDB, the pH media is adjusted in the range of 3, 4, 5, 6, 7, 8 in static conditions, incubated at room temperature. After 7th days, the culture fluid adjusted to neutral pH, and examined the impact of resistance to \textit{S. aureus} and MRSA by disc diffusion method. [3]

2.2.3. Screening for endophytic fungi antibacterial metabolites

Endophytic fungi culture on PDA from 5-12 days at room temperature were tested for the antibacterial activity against \textit{Staphylococcus aureus}, \textit{E. coli}, \textit{Streptococcus faecalis}, MRSA by disc diffusion method.

\textbf{Antibacterial assay:} The antibacterial activity of the secondary metabolites which were extracted from the culture of endophytes were determined by disc diffusion method. The suitable conditions for endophytes produced antibacterial substance were determined basing on diameter of inhibition zone.

\textbf{Incubation time on the production of antibacterial substance of N-GL1:} N-GL1 were cultured on PDB with the initial amount $10^4$ CFU/L, glucose 2% (w/w), potatoes 20% (w/v), incubated at room temperature for static conditions. Monitor antimicrobial substances produced by fungi into the culture medium over time by means of diffusion through after 5, 7, 9, 12 days.

2.2.4. The effect of carbon source, nitrogen source, aeration, oil on the production of antibacterial substances by Design-Expert 6.0.6 Software

N-GL1 were cultured as designed in the same conditions, the amount of the initial spore $10^4$ CFU/ml. Determine to the antibacterial substances produced by fungi in culture media from time to time by disc diffusion method. The culture conditions were determined based on diameter of inhibited zones [2],[5], [8], [11].

Vegetable oil was used to supplement the carbon source on the production of antibacterial substances. N-GL1 was determined the composition and culture conditions for high antibacterial activity with the carbon source was molasses 2.7% were added 1% sesame oil, 1% sunflower oil, 1% soybean oil, 1% corn oil, 1% olive oil. Survey antibacterial action \textit{S. aureus} and MRSA of culture fluid [6], [10].

2.2.5. Surveying the optimal culture conditions

Process design and basic environmental survey includes 5 main stages as follows:

1. Design with experimental models Design- Expert 6.0.6 software
2. Empirical model D - Optimal Formula 26
3. The independent variables \( x_i \) (environmental component)
4. The dependent variable \( y_i \)
5. \( A. \ terreus \) were cultured as designed in the same conditions and processes at room temperature, static conditions. Evaluated of results in antibacterial of culture fluid and biomass obtained at day 7 by means of diffusion through wells. [8]

2.2.6. The optimal culture conditions
Data input is the environment and the value parameters \( y_i \) of recipes 26. Environmental optimization is done by BC Pharsoft software. Verify the optimal environment, 3 batches cultured under optimal culture conditions. Culture fluid was determined the antibacterial diameter and similar biomass obtained in environmental design stage to compare the results from the software. [1],[8]

3. Results and discussions
3.1. Identification of \( N-GL1 \) strain
Fungal identifications were based on the morphological characteristics. \( N-GL1 \) strain was identified as strains of \( A. \ terreus \): Features of fungal colonization on PDA culture medium: fast development speed \( \Phi = 6 \) cm/7 days. Finely powdered mycelia, took the turn light brown to dark brown over time, golden brown on the underside, diffused yellowish brown pigment into the environment. (Fig.1)

![Figure 1](image)

**Figure 1.** The strain of \( A. \ terreus \) N-GL1
(A. Colony of N-GL1 on PDA medium, B. Sterigmta, C. Conidia)

3.2. Effect of pH on the Production of Antibacterial Production of N-GL1 Strain
The pH of culture medium is one of the determinant factors for the microbial growth, pigment production and biosynthesis of secondary metabolites. \( N-GL1 \) strain was grown at different initial pH values (3.0 to 8.0) in static conditions. The pH 7.0 was suitable for the maximum production of antibacterial agent. (Table 1)

| pH media | Diameter of inhibited zones (mm) |
|----------|----------------------------------|
|          | MRSA | \( S. \) aureus               |
| 3        | 0    | 0                              |
| 4        | 0    | 0                              |
| 5        | 0    | 0                              |
| 6        | 16   | 16                             |
| 7        | 18   | 18                             |
| 8        | 17   | 17                             |

Table 1. The production of antibacterial substances of \( A. \ terreus \) N-GL1 strain cultured in different pH media
3.3. Effect of incubation time on the production of antibacterial activity of N-GL1 strain

After seven days incubated at room temperature, A. terreus N-GL1 strain can produce the substances against *S. aureus* and MRSA in higher level than other days. (Table 2).

| Incubation time (days) | Diameter of inhibited zones (mm) |
|------------------------|----------------------------------|
|                        | MRSA                | *S. aureus*        |
| 5                      | 12                  | 12                  |
| 7                      | 19                  | 19                  |
| 9                      | 18                  | 18                  |
| 12                     | 16                  | 14                  |

3.4. Effect of carbon sources, nitrogen sources, aeration, oil on the production of Antibacterial Substances from A. terreus N-GL1

Based on the basic medium with nitrogen source was potatoes 20%, carbon source was glucose 2%, changes nitrogen and carbon source base on equivalent to the nitrogen content of potatoes 20% and glucose 2%. Concentration of carbon and nitrogen sources were presented in Table 3. Using the Design Expert 6.0.6 software to design experiments with x1 variable was source of nitrogen, x2 was carbon source, x3 was ventilation conditions (shaking 200 cycles/min or static). The amount of input is 104CFU/ml, incubation at room temperature. The culture conditions were identified based on diameter of inhibited zones of *S. aureus* and MRSA after 5, 7, 9, 12 days.

In the medium components, carbon sources, nitrogen sources and aeration conditions were changed by independent variables. The survey by the independent variables are presented in the following table:

| Level | x1          | x2          | x3          |
|-------|-------------|-------------|-------------|
| 5     | Glucose 2%  | Potatoes 20%| Shaking     |
| 7     | Saccharose 1%| Peptone 1%  | Static      |
| 9     | Rice starch 2%| Yeast extract 1% |            |
| 12    | Molasses 2.7%| Soybean 10% |             |

An empirical model was designed by the Design-Expert 6.0.6 software includes environments 23. Environmental data and diameter of inhibited zones (mm) is presented in Table 4. Twenty-three environments were cultured in the same process and experimental conditions.

| MT  | X1          | X2          | X3          | Diameter of inhibited zones (mm) |
|-----|-------------|-------------|-------------|----------------------------------|
|     |             |             |             | *S. aureus* | MRSA                              |
| 1   | Peptone     | Molass      | Shaking     | 5          | 7          | 9          | 12         | 5          | 7          | 9          | 12         |
| 2   | Soybean     | Molass      | Static      | 0          | 0          | 0          | 0          | 0          | 0          | 0          | 0          |
| 3   | Peptone     | Rice starch | Static      | 13         | 16.5       | 14         | 13         | 12         | 18         | 14         | 14         |
| 4   | Soybean     | Rice starch | Static      | 11         | 15         | 12         | 13         | 11         | 14         | 12         | 12         |
| 5   | Soybean     | Saccharose  | Static      | 0          | 0          | 0          | 0          | 0          | 0          | 0          | 0          |
| 6   | Peptone     | Saccharose  | Static      | 0          | 0          | 0          | 0          | 0          | 0          | 0          | 0          |
| 7   | Potato      | Glucose     | Static      | 12         | 19         | 18         | 14         | 12         | 19         | 18         | 16         |
From the 23 culture mediums and different conditions, with culture concentrations were potatoes 20%, molasses 2.7% or saccharose 1%, in static conditions. The result show that the activity of A. terreus N-GL1 resistant MRSA and S. aureus highly; appropriate time to acquire antimicrobial from culture fluids after 7 days.

Impact of oil on the production of antibacterial substances of A. terreus N-GL1: Surveying the influence of oil on the production of antibacterial substances of A. terreus N-GL1, vegetable oils such as sesame oil, sunflower oil, soybean oil, corn oil, olive oil (1% concentration) was added culture media with ingredients including potatoes 20%, molasses 2.7%, in static conditions from culture fluids after seventh days by disc diffusion method. Implemented in parallel with culture fluid of the same composition and culture conditions without additional vegetable oil. (Table 5).

| MT | X1 | X2 | X3 | Diameter of inhibited zones (mm) |
|----|----|----|----|---------------------------------|
|    |    |    |    | S.aureus | MRSA |
| 5  | 7  | 9  | 12 | 5     | 7   | 9  | 12 |
| 8  | Peptone | Glucose | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9  | Soybean | Glucose | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | Potato | Rice starch | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | Potato | Molass | Static | 17 | 23 | 18 | 19 | 16 | 23 | 19 | 19 | 0 | 0 | 0 | 0 |
| 12 | Yeast extract | Glucose | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | Yeast extract | Saccharose | Static | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | Yeast extract | Rice starch | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Peptone | Glucose | Static | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Potato | Saccharose | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | Soybean | Molass | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | Yeast extract | Molass | Static | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | Potato | Saccharose | Static | 16 | 23 | 14 | 15 | 11 | 23 | 16 | 15 | 0 | 0 | 0 | 0 |
| 20 | Soybean | Glucose | Static | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | Yeast extract | Saccharose | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | Yeast extract | Molass | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | Peptone | Rice starch | Shaking | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 5. The production of antimicrobial substances of A. terreus N-GL1 on carbon sources, nitrogen sources, different ventilation conditions

| Incubation culture | Diameter of inhibited zones (mm) |
|--------------------|---------------------------------|
|                    | S.aureus | MRSA |
| Potato-Molass      | 20       | 19   |
| Potato-Molass + sesame oil | 15     | 15   |
| Potato-Molass + sunflower oil | 16    | 15   |
| Potato-Molass + soybean oil | 18    | 17   |
| Potato-Molass + corn oil | 17     | 17   |
| Potato-Molass + olive oil | 15     | 16   |

Oil-supplemented culture medium have not impact on the production of antibacterial substances by A. terreus N-GL1.

3.5. Design and optimization of culture conditions

Experimental models were designed in software Design - Expert 6.0.6 with the following variables:

- $x_1$: Potatoes concentration 10%, 20%, 30%.
- $x_2$: Glucose concentration of carbon sources: molasses 2%, 3%, 4%; saccharose 0.5%, 1%, 2%.
- $x_3$: Carbon source is molasses, saccharose
- $x_4$: The amount included in the initial yeast with OD: 0.05; 0.1; 0.2.
The survey by the independent xi variable was presented in Table 6.

**Table 6.** Survey the empirical model to find the optimal environmental elements

| Level | x₁ | x₂ | x₃ | x₄ |
|-------|----|----|----|----|
| 1     | 10%| 2  | Molass | 0.05 |
| 2     | 20%| 3  | Molass | 0.05 |
| 3     | 30%| 4  | Molass | 0.05 |
| 4     | 0.5| Saccharose | 0.05 |
| 5     | 1  | 1  |         | 0.05 |
| 6     | 2  | 2  |         | 0.05 |

Empirical model designed by Design-Expert 6.0.6. software includes 26 environmental. Environmental data, the antibacterial diameter and biomass were presented in Table 7. Twenty-six environments were cultured in the same process and the experimental conditions.

**Table 7.** Culture media of *A. terreus* N - GL1 was designed by Design Expert 6.0.6 software

| Number | x₁ | x₂ (%) | x₃ | x₄ | y₁ | y₂ |
|--------|----|--------|----|----|----|----|
| 1      | 20 | 2      | Molass | 0.05 | 14.5 | 1.9 |
| 2      | 10 | 2      | Molass | 0.05 | 18  | 0.8 |
| 3      | 10 | 2      | Molass | 0.1  | 20.5 | 1.3 |
| 4      | 30 | 2      | Molass | 0.2  | 14  | 3.5 |
| 5      | 30 | 2      | Molass | 0.05 | 15  | 2.9 |
| 6      | 10 | 3      | Molass | 0.2  | 21  | 0.6 |
| 7      | 30 | 3      | Molass | 0.05 | 15  | 3.7 |
| 8      | 10 | 3      | Molass | 0.1  | 21.5| 1  |
| 9      | 20 | 3      | Molass | 0.2  | 16  | 1.9 |
| 10     | 30 | 4      | Molass | 0.1  | 15  | 2.1 |
| 11     | 20 | 4      | Molass | 0.05 | 16  | 1.3 |
| 12     | 10 | 4      | Molass | 0.2  | 21  | 0.8 |
| 13     | 20 | 4      | Molass | 0.1  | 16  | 0.9 |
| 14     | 20 | 0.5    | Saccharose | 0.2 | 13  | 1  |
| 15     | 10 | 0.5    | Saccharose | 0.05 | 20  | 1.4 |
| 16     | 30 | 0.5    | Saccharose | 0.1 | 14.5| 1.3 |
| 17     | 10 | 0.5    | Saccharose | 0.2 | 17  | 0.7 |
| 18     | 20 | 0.5    | Saccharose | 0.1 | 13  | 0.1 |
| 19     | 30 | 1      | Saccharose | 0.1 | 16  | 1.9 |
| 20     | 30 | 1      | Saccharose | 0.2 | 14  | 0.7 |
| 21     | 20 | 1      | Saccharose | 0.05 | 14  | 1.4 |
| 22     | 10 | 1      | Saccharose | 0.05 | 19  | 1.4 |
| 23     | 10 | 2      | Saccharose | 0.1 | 14  | 0.9 |
| 24     | 30 | 2      | Saccharose | 0.05 | 14  | 3  |
| 25     | 10 | 2      | Saccharose | 0.05 | 13.5| 0.3 |
| 26     | 20 | 2      | Saccharose | 0.2 | 15  | 2.6 |

Independent variables: x₁, x₂, x₃, x₄ (With: x₁: potatoes concentration; x₂: carbon concentration; x₃: carbon source, x₄: The amount included in the original fungi).
Dependent variables: y₁, y₂ (y₁: Diameter antibacterial (mm); y₂: Biomass (g)).
3.6. Optimize Culture Conditions
Data in table 7 was used as input to the Design Expert software to optimize the parameters of the environment.

| Algorithm            | R² training | R² test |
|----------------------|-------------|---------|
| BackPropagation Learning | 0.99        | 0.99    |
| BackPropagation Learning | 0.99        | 0.95    |

Results of R² training and R² test and try to find an association between components causal formula with properties parameters were reviewed. Results optimized by intelligent software includes BC Pharsoft optimal parameters of culture conditions and the predicted value of the product properties are as follows:
Optimal composition formula: \( y_1 = 21.344; y_2 = 2.608 \); Optimal environmental elements include potatoes predicted 10%; molasses 2.2%, \( \text{OD}_{530\text{nm}} = 0.14 \) (1 ml fungal to 100 ml cultures); Verify the optimal recipe empirically, compared with predicted results 3 batch cultures conducted with optimal conditions in the same conditions and determine the parameters \( y_i \). (Table 9); Process repetitive culture and nature products between 3 different batches without statistical significance \((p = 0.87 > 0.05)\).

| Lot      | Product characteristics |
|----------|-------------------------|
|         | \( y_1 \) | \( y_2 \) |
| Lot 1   | 22         | 0.46 |
| Lot 2   | 21         | 0.64 |
| Lot 3   | 21         | 0.62 |
| Average | 21.33      | 0.57 |
| Predict | 21.344     | 2.608 |

The result is the average of 3 lots of culture and anticipated results of the different software without statistical significance \((p > 0.05)\). Therefore, the BC Pharsof software has correctly predicted optimal culture conditions.

4. Conclusions
This study have surveyed the factors for \( A. \text{ terreus N-GL1} \) produce secondary metabolites against \( S. \text{ aureus} \) and MRSA (pH media, vegetable oil, carbon source, nitrogen source, ventilation conditions and incubation time). The results show that in 23 test culture medium with different sources of nitrogen and carbon, \( A. \text{ terreus N-GL1} \) for antibacterial substances highest in culture media as nitrogen source was potatoes, carbon source was saccharose or molasses. Using software components Design Expert 6.0.6 and BC pharsoft predicted optimal culture for \( A. \text{ terreus N-GL1} \) produce antibacterial substances highest: nitrogen source was potatoes 10%; source of carbon was molasses 2.2%; initial amount of yeast cells was \( 10^4 \text{ CFU/ml} \); incubated in static conditions for seventh days. Identifying antibacterial efficacy by diffusion method showed that the experimental results fit predictions of software.

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