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RESEARCH ARTICLE

REVISED Optimization of glutaminase-free L-asparaginase production using mangrove endophytic *Lysinibacillus fusiformis* B27 [version 2; peer review: 2 approved]

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Abstract

Background: The mangrove, *Rhizophora mucronata*, an essential source of endophytic bacteria, was investigated for its ability to produce glutaminase-free L-asparaginase. The study aimed to obtain glutaminase-free L-asparaginase-producing endophytic bacteria from the mangrove and to optimize enzyme production.
Methods: The screening of L-asparaginase-producing bacteria used modified M9 medium. The potential producer was further analyzed with respect to its species using 16S rRNA gene sequencing. Taguchi experimental design was applied to optimize the enzyme production. Four factors (L-asparagine concentration, pH, temperature, and inoculum concentration) were selected at four levels.
Results: The results indicated that the endophytic bacteria *Lysinibacillus fusiformis* B27 isolated from *R. mucronata* was a potential producer of glutaminase-free L-asparaginase. The experiment indicated that pH 6, temperature at 35°C, and inoculum concentration of 1.5% enabled the best production and were essential factors. L-asparagine (2%) was less critical for optimum production.
Conclusions: L. fusiformis B27, isolated from *Rhizophora mucronata*, can be optimized for L-ASNase enzyme production using optimization factors (L-ASNase, pH, temperature, and inoculum), which can increase L-ASNase enzyme production by approximately three-fold.

Keywords

L-asparaginase, mutation, statistical, taguchi, Lysinibacillus fusiformis.

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Introduction

L-asparaginase (L-ASNase, EC.3.5.1.1, L-asparagine amido-hydrolase) hydrolyzes L-asparagine (L-ASN) to L-aspartic acid and ammonia[1]. This enzyme has an essential role in food safety owing to its acrylamide-mitigating potential[2,3]. Acrylamide, a compound formed from the reaction of asparagine with reducing sugars, can be converted into L-asparagine (ASN) in raw materials by adding L-ASNase. The food industry mainly uses two commercial products, Acrylaway and Preventase from *Aspergillus oryzae* and *Aspergillus niger*, respectively[4,5].

L-ASNase can also inhibit the growth of cancer cells, especially leukemia[4], and it is potentially suitable for leukemia treatment due to its ability to deplete L-ASN in blood plasma[5]. Cells in healthy tissue can synthesize L-ASN in sufficient amounts for protein synthesis, but some types of lymphoid malignancies remove it from plasma. Limiting the amount of L-ASN will eventually inhibit the growth of cancer cells. Applying L-ASNase to the treatment of acute lymphoblastic leukemia, acute myelocytic leukemia, Hodgkin's disease, non- Hodgkin's disease, and melanosarcoma has been investigated for its effectiveness in animals and humans[6].

Some characteristics of this enzyme hinder its application in foods and medicines. For example, it is unsuitable for application in foods due to its sensitivity to high temperatures[6]. In medical applications, it has a low half-life and can cause allergic reactions in patients[6]. Some of these operational and technical constraints cause manufacturers to identify new sources of L-ASNase. Therefore, new L-ASNase producers need to be explored.

L-ASNase is commonly found in animal tissues, bacteria, and plants, but is lacking in humans. L-ASNase is produced in large quantities by several microorganisms such as *Enterobacter aerogenes, Escherichia coli, Erwinia carotovora, Enterobacter aerogenes, Candida sp.,* and *Corynebacterium glutamicum*[7,8]. Production of L-ASNase sourced from animals or plants encounter several impediments, including those relating to large-scale enzyme production. Therefore, the production of L-ASNase derived from microorganisms is preferred because these multiply rapidly and are easily managed[9].

Optimizing the growth parameters improves enzyme production. Enzyme production can be optimized via a statistical approach using Taguchi analysis. The Taguchi method facilitates product or process design[10]. This approach offers time efficiency and accurate results, using relatively few treatment parameters[11].

Considering the drawbacks of the L-ASNase toward medical and food application, and for fulfilling the need for L-ASNase with better properties, The search for exploring the new source of L-ASNase is remarkably important. This study was conducted to isolate and identify potential endophytic bacteria producing the L-ASNase enzyme from the mangrove *Rhizophora mucronata*. Optimization of L-ASNase enzyme production by selected bacteria was performed by optimizing factors influencing production (L-ASN, pH, temperature, inoculum concentration, and incubation time) using a statistical Taguchi approach.

Methods

Isolation and screening of L-ASNase producers

Isolation and screening of L-ASNase-producing endophytic bacteria was performed as described by Prihanto *et al.*[12] and Mahajan *et al.*[13]. Mangrove *(Rhizophora mucronata)* was obtained from Aeng Sareh Beach, Madura Island, Indonesia with the location of 7°13'5.57"S and 113°19'8.89"E. Mangrove stem was aseptically cut to approximately 1 cm diameter. The sample was placed in a plastic bag and immediately transported to the laboratory on ice (4°C). It was crushed and weighed (1 g) and then serially diluted with physiological buffer. Three independent treatments were applied. An appropriately diluted sample (10^{-3}–10^{-5}) was plated onto Luria-Bertani agar (Sigma-Aldrich, USA) using the pour plate method and incubated for 72 h at 35°C. Colonies were purified in Luria-Bertani agar (Sigma-Aldrich, USA) using three quadrants streaking, and stored at –20°C.

A modified M9 medium was used for screening isolates that were produced glutaminase-free L-ASNase. All materials and reagents were purchased from Merck, USA. The composition of the medium was 6 g/l of Na_2HPO_4, 2 g/l of KH_2PO_4, 0.5 g/l of NaCl, 20 g/l of L-ASN or L-glutamine, 2 g/l of glucose, 0.2 g/l of $MgSO_4$, 0.005 g/l of $CaCl_2$, agar 2%, and Bromotymol blue (BTB) 0.007%. The pH of the medium was set to 5.5 using a pH meter with 2 N HCl. BTB served as a color indicator. Two media (M9 medium with L-ASN, and M9 medium with L-glutamine) were applied to the isolates. Glutaminase-free L-ASNase isolates were detected by the ability to hydrolyze only L-ASN.For isolate which able to hydrolyze both L-ASN, and L-glutamine was not chosen for further analysis. Initial identification of the selected isolates was performed by Gram staining.

Identification of bacterial isolates

Molecular identification of the selected bacterial isolates from mangrove was performed based on the methods of Prihanto *et al.*[12]. Genomic DNA from bacteria was amplified using 27F primers (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGA-3'). Primers were purchased from 1st BASE (Singapore). Genomic DNA of bacteria were extracted, and amplified by following the company manual procedures (Wizard™ DNA purification Kit, Cat. No. A1120, and Gotaq® DNA polymerase, Cat No. M3005, Promega, USA). Obtained DNA sample (1 μL) was mixed with 18.5 μL double

distilled H_2O (ddH_2O), 2.5 µL Buffer B with Mg2 + 10X, 1 µL dNTPs, 1 µL forward primer, 1 µL reverse primer, and 0.2 µL Taq polymerase. The reaction was performed in a thermocycler (T100 Thermal Cycler, Bio-Rad, USA) (denaturation: 94°C, 45 s; annealing: 61°C, 45 s; elongation: 72°C, 2 min). The amplification process was performed over 32 cycles. The Polymerase Chain Reaction results were checked by gel electrophoresis (Mupid EXu submarine, Takara, Japan) under 80 volt and 40 mA with 1.5% agarose. The gel was visualized using Benchtop UV Transilluminator (UVP, Canada).

The amplicon was further sequenced with ABI PRISM 3130×1 DNA sequencer (Applied Biosystems, USA). The sequence homology was investigated at the National Center for Biotechnology Information (NCBI) GenBank database using BLAST nucleotide programs, on the nucleotides collection database by excluding human and mouse genomic. The program was optimized for highly similar sequences (megablast). A phylogenetic tree was constructed using the UPGMA method with the MAFFT online ver 7. phylogenetic analysis service[14].

Optimization of L-ASNase production

All materials and reagents were purchased from Merck, USA. Enzyme production was performed in a medium containing trisodium citrate 0.375 g, $(NH_4)_2HPO_4$ 0.1 g, K_2HPO_4 0.00625 g, $MgSO_4 \cdot 7H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.001 g, yeast extract 0.075 g, and L-ASN 0.25–1 g[15]. Different volume of inoculum (10^8 CFU/ml) was inoculated on 50 ml fresh treated-production mediums. The cultures were incubated in a shaker incubator (120 rpm) at different temperatures. Four factors at four levels, L-ASN (0.5–2%), pH (6–9), temperature (30–45°C), and inoculum volume (0.5–2%), were investigated (Table 1). The L16 orthogonal array was selected for experimental design (Table 2). After 24 h incubation, the culture was harvested and the production of enzyme was investigated.

L-ASNase assay

A slightly modified method[16] was used for the enzyme activity assay. The samples (150 µl) were transferred to microtubes to which 100 µl of 150 mM L-ASN, 200 µl aquades, and 50 µl of 1 M phosphate buffer (pH 7) were added before homogenization. The samples were then incubated in a water bath at 30°C for 30 min to react. Subsequently, 100 µl of 1.5 M Trichloro Acetic Acid was added to stop the reaction followed by centrifugation at 2,000 rpm for 12 min; the supernatant

Table 2. Orthogonal array of designed experiment (L16).

Exp. No.	Factor level				L-ASNase activity (U/ml)
	L-ASN	pH	Temp	Inoculum	
1	1	1	1	1	3.32±0.03
2	1	2	2	2	15.43±0.25
3	1	3	3	3	0.42±0.08
4	1	4	4	4	12.02±1.30
5	2	1	2	3	11.56±2.08
6	2	2	1	4	3.68±0.01
7	2	3	4	1	10.83±0.57
8	2	4	3	2	5.02±0.06
9	3	1	3	4	6.47±1.57
10	3	2	4	3	9.11±0.33
11	3	3	1	2	6.74±0.08
12	3	4	2	1	11.45±0.91
13	4	1	4	2	7.09±0.08
14	4	2	3	1	1.09±0.04
15	4	3	2	4	16.04±1.38
16	4	4	1	3	4.15±0.34

(450 µl) was then transferred into the microtube. Nessler's reagent (125 µl) was then added and reacted for 15 min until the color changed; the absorbance was read with a UV–vis spectrophotometer at a wavelength of 480 nm. NH_4Cl served as standard ammonia. The enzyme activity was expressed as micromoles ammonia released per minute.

The activity was calculated with the following formula:

$$\text{Enzyme activity (U/ml)} = \frac{(\mu\text{mol of liberated NH3}) x \, (0.6)}{(0.45) \times (30) \times (0.1)}$$

Where 0.6 = initial volume of enzyme mixture (ml)

    0.45 = volume of enzyme mixture used in final reaction (ml)

    30 = incubation time (min)

    0.1 = volume of enzyme used (ml)

Data analysis

All L16 data were analyzed using Qualitek-4 software (Nutek Inc., MI). The analysis was used to obtain the predicted optimum conditions for achieving the highest L-ASNase production. Analysis of variance (ANOVA) was used to identify factors that influenced L-ASNase production. The F-ratio was calculated with a 95% confidence interval. Interactions among factors and levels of experiment was analyzed by determining the severity index (SI)

Experimental validation was conducted to confirm the accuracy of the obtained results. This aimed to verify the optimum factors and levels obtained from the experiment. After determining the optimum treatment, the next step was to conduct a confirmatory experiment by creating the optimum conditions based on the predetermined factors and levels.

Table 1. Selected factors and levels for L-asparaginase production.

Factors	Level			
	1	2	3	4
L-asparagine	0.5%	1%	1.5%	2%
pH	6	7	8	9
Temperature	30°C	35°C	40°C	45°C
Inoculum	0.5%	1%	1.5%	2%

Results and discussion

Screening and identification

The screening results showed that 31 endophytic mangrove isolates could produce the L-ASNase enzyme. Judging from the blue color of the medium, isolate B27 was the best producer (Figure 1A). Isolate B27 had a more extensive and darker blue tone compared to other isolates. Furthermore, only isolate B27 produced glutaminase-L-asparaginase. Hence, further molecular identification was applied only to the B27 isolate. Further, Gram

stain analysis revealed that the bacteria was Gram positive (Figure 1B).

The 16S rDNA molecular identification results showed that B27 isolates had a similarity of 98% with the *Lysinibacillus fusiformis* species. The phylogenetic analysis results showed that *L. fusiformis* was most closely related to *L. fusiformis* strain NBRC15717 (Figure 2). The production of L-ASNase from this bacteria was further optimized using the Taguchi method.
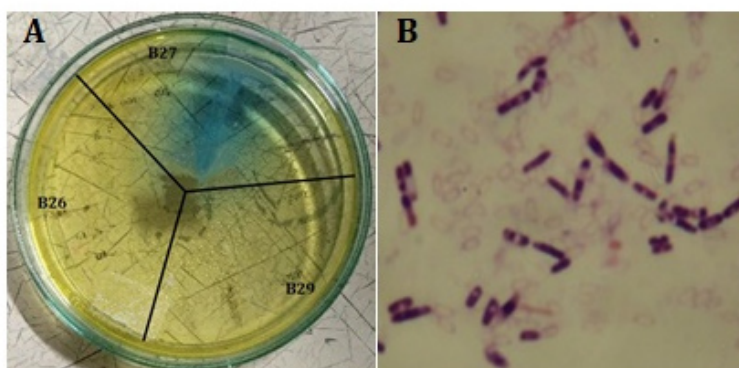

Figure 1. Results of L-asparaginase screening producing endophytic bacteria B27 isolated from *Rhizophora mucronata*. (**A**) Blue color indicating the production of glutaminase-free L-asparaginase. (**B**). Gram stain of B27 isolate.
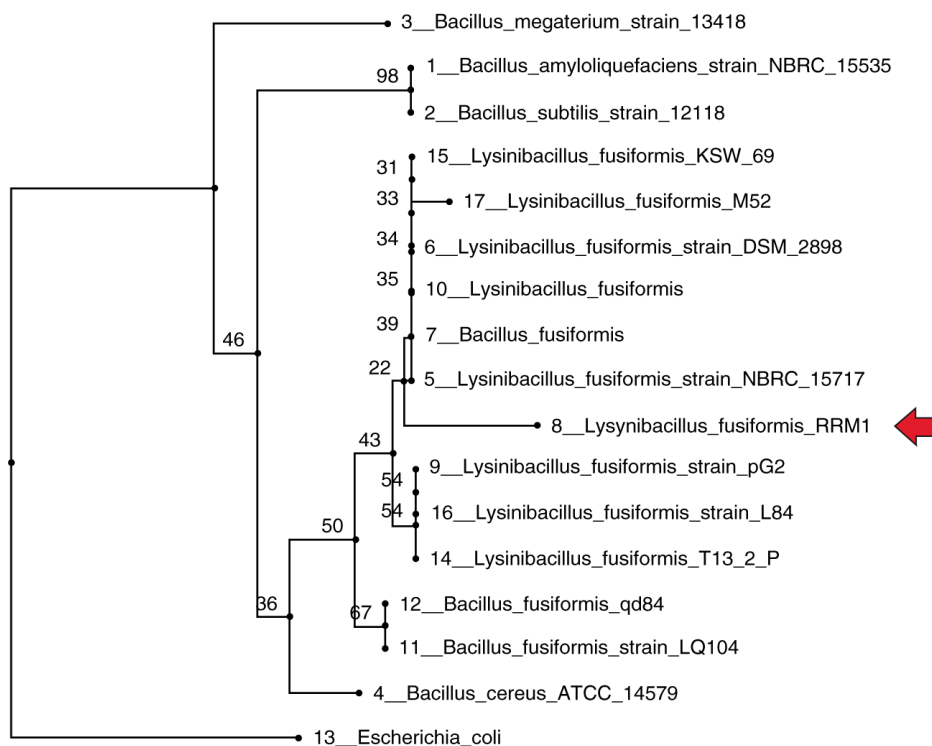

Figure 2. UPGMA phylogenetic tree of *Lysinibacillus fusiformis* B27 isolated from *Rhizophora mucronata*.

Optimization of L-ASNase production

L-ASNase production was controlled to determine enzyme production without optimization. The enzyme production during incubation periods of 24 and 48 h was 3.18 and 1.99 U/ml, respectively. The higher production was observed after 24 h. During the exponential phase, bacteria experience fast growth and produce metabolites for growth and self-defense[17]. The result for optimization experiment is shown in Table 2.

Influence of culture factors

To determine the effects of factors that could increase the L-ASNase production, L-ASN concentration (0.5, 1, 1.5, and 2%), pH (6, 7, 8, and 9), temperature (30, 35, 40, and 45°C), and inoculum concentration (0.5, 1, 1.5, and 2%) were evaluated. L-ASN concentration was used to determine the effect of inducers on increasing enzyme production. pH, temperature, and inoculum concentration were subsequently investigated. The effects of these factors are shown in Figure 3.

L-ASN concentration influenced L-ASNase production, with the best concentration being 2% L-ASN with enzyme production of 9.466 U/ml. Therefore, to some extent, L-ASN could increase enzyme activity. However, the results showed that L-ASN concentration 1% could reduce enzyme production. Consequently, the higher the substrate concentration added to the enzyme solution, the lower the enzyme activity until the enzyme reached the optimum substrate concentration[18]. This data suggested that the enzyme production exhibited an optimum concentration of more than 2% L-ASN in the medium.

L. fusiformis B27 at pH 6 showed an enzyme production of 9.795 U/ml. When the pH was too high or low, enzyme production was reduced. Therefore, the optimum pH for enzyme production must always be considered. *L. fusiformis* B27 grown at 35°C showed the highest enzyme production of 17.037 U/ml. Temperature plays a vital role in enzymatic reactions. High temperatures increase the reaction rate of the
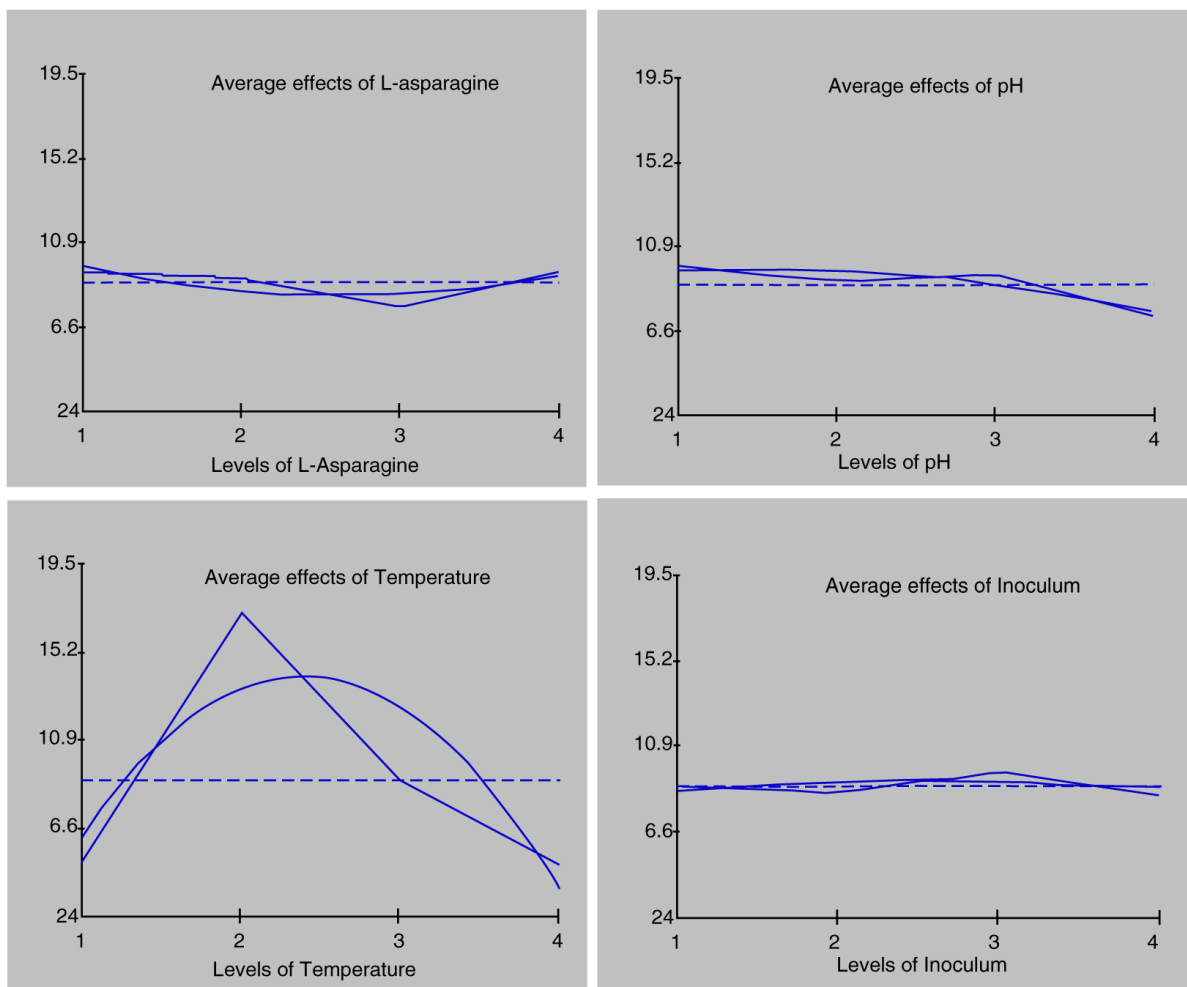

Figure 3. Impact of selected variables (L-asparagine, pH, temperature and inoculum) on the production of L-asparaginase producing bacteria isolated from *Rhizophora mucronata*. Angled lines is the enzyme activity. Non-angled lines is regression function of the enzyme activity.

enzyme. Hence, the optimum temperature for enzymes needs to be determined.

The optimum inoculum concentration for L-ASNase production was 1.5% with an enzyme production of 9.638 U/ml. A very high inoculum concentration can cause competition between microbes in obtaining nutrition. This competition can cause some microbes to lack the nutrients required for growth[19,20].

Factors influencing enzyme production

Analysis of variance (ANOVA) was used to identify factors that influenced L-ASNase production (Table 3). The F-ratio was calculated with a 95% confidence interval. The results showed that all factors significantly influenced enzyme production. The most influential factor affecting L-ASNase enzyme production was temperature, while the inoculum concentration, although it was known to affect the enzyme production, showed the least effect.

Factor interaction, optimized conditions, and validation of enzyme production

Interactions among factors and levels of experiment can be analyzed by determining the severity index (SI), as shown in Table 4. The SI can be used to predict the effect of combined factors on enzyme production.

The SI measures the interaction between two factors. The analysis showed that the interaction between pH and inoculum was the highest with a SI of 69.72%, while the lowest SI of 4.5% was found for the interaction between pH and temperature. The interaction between pH and inoculum significantly influenced L-ASNase production. Conversely, the ANOVA results showed that the most influential factor for L-ASNase

production was temperature. Different SI values could result in different values of individual factors[21].

Optimum factors for enzyme production

L-ASN at a concentration of 2% (level 4), pH 6 (level 1), temperature 35°C (level 2), and an inoculum concentration of 1.5% (level 3) for 24 h of incubation were deduced as the best conditions for L-ASNase production with an expected enzyme production of 19.2065 U/ml (Table 5).

A validation experiment was required to confirm the expected result. Based on the confirmation test results, the L-ASNase activity obtained from *L. fusiformis* B27 at 24 h was 8.51 U/ml. The L-ASNase enzyme activity from *L. fusiformis* B27 at 24 h before and after optimization is shown in Table 6.

The data showed that before optimization (control) L-ASNase had a low activity of 3.18 U/ml. This was because the bacterial environment for producing L-ASNase was suboptimal. The L-ASNase activity increased to 8.51 U/ml after optimization. The optimized factors for enzyme production could be predicted using the Taguchi method[22–24]. Confirmation experiment results are usually closer to the predicted values. However, these are sometimes lower than the predicted results. In this study, the optimized factors enhanced the enzyme production from 3.18 to 8.51 U/ml. This represented an almost three-fold increase. This increase was higher than that published in previous research[21] but lower than that in another study[25].

Conclusions

Lysinibacillus fusiformis B27, which was isolated from mangrove, *Rhizophora mucronata,* can be optimized for L-ASNase enzyme production using optimization factors (L-ASNase, pH,

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Table 3. ANOVA analysis for investigated factors.

Factor	DOF (f)	Sum of squares	Variance (V)	F-Ratio (F)	Pure sum (S')	Percent P (%)
L-asparagine	3	16.195	5.,398	2.633	10.046	1.141
pH	3	27.906	9.302	4.538	21.757	2.472
Temperature	3	790.709	263.569	128.592	784.56	86.148
Inoculum	3	6.309	2. 103	1.026	0.16	0.018
Other/Error	19	38.942	2.049			7.221

Table 4. Estimated interaction of severity index.

Interacting factor pairs	Columns	SI (%)	Col	Opt.
pH x Inoculum	1 × 2	69.72	3	2.1
L-asparagin x temperature	2 × 4	21.86	6	1.3
L-asparagin x Inoculum	1 × 4	14.84	5	2.3
L-asparagin x pH	1 × 3	13.7	2	2.2
temperature x Inoculum	3 × 4	13.38	7	2.3
pH x temperature	2 × 3	4.5	1	1.2

Table 5. The predicted factors for optimum production of L-asparaginase.

Factor	Level description	Level	Contribution
L-asparagine	2%	4	0.556
pH	6	1	0.885
Temperature	35°C	2	8.127
Inoculum	1.5%	3	0.729
Total contribution from all factors			10.269
Current Grand Average of performance			8.909
Expected Result at Optimum condition			19.206

Table 6. Comparison of enzyme production.

Experiment	Enzyme production
Control	3.18 U/ml
Expected result	19.20 U/ml
Validation result	8.51 U/ml

temperature, and inoculum). Optimization using the Taguchi approach can increase L-ASNase enzyme production by approximately three-fold.

Data availability

Underlying data

Figshare: Raw data for production improvement of L-Asparaginase from *Lysinibacillus fusiformis*. https://doi.org/10.6084/m9.figshare.10265396.v2[26]

This project contains the following underlying data:

- L-ASNase activity for Taguchi analysis for 16 optimization experiments.

- Results for confirmatory experiment

- Images of all 31 isolates

- Uncropped, unedited blots from genome (B27)

- Sequence of B27

- Uncropped, unedited image of Gram stain of B27, as shown in Figure 1B

Acknowledgements

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    PubMed Abstract | **Publisher Full Text** | **Free Full Text**

26. Prihanto AA, Yanti I, Murtazam MA, *et al.*: **Raw Data (L-ASNase activity for Taguchi analysis).xlsx.** *figshare.* Dataset. 2019.
    http://www.doi.org/10.6084/m9.figshare.10265396.v2

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 10 June 2020

✔ **Evans Manyara Nyakeri** (iD)

Africa Centre of Excellence in Sustainable Use of Edible Insects as Food and Feeds (INSEFOODS) and School of Agricultural and Food Sciences, Jaramogi Oginga Odinga University of Science and Technology, Bondo, Kenya

I have reviewed the corrections made by the authors against the suggestions I had made earlier and it is my conclusion that they have been addressed satisfactorily. I, therefore, approve the revised form of the manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, biotechnology, food security and applied insect science.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 01 June 2020

✔ **Sathyanarayana N. Gummadi** (iD)

Applied and Industrial Microbiology Lab, Department of Biotechnology, Indian Institute of Technology Madras, Chennai, India

The authors addressed all the queries except for the comment about the novelty:

The values reported in this work are very low compared to commercial enzymes and other

published reports. Authors claim that among endophytes this strain seems better. Authors need to justify in the introduction why L-asparaginases from endophytes are required when compared to E.coli and other bacterial and fungal strains.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Version 1

Reviewer Report 07 May 2020

https://doi.org/10.5256/f1000research.23313.r62909

? **Sathyanarayana N. Gummadi** (iD)

Applied and Industrial Microbiology Lab, Department of Biotechnology, Indian Institute of Technology Madras, Chennai, India

The study investigated the ability of mangrove *Rhizophora mucronata* to produce glutaminase-free L-asparaginase and then production enhancement by optimizing parameters L-asparagine concentration, pH, temperature, and inoculum concentration by Taguchi method.

Numerous reports are available on glutaminase-free asparaginase producing microbial sources and considering this, results presented in this study are too preliminary and authors should think the novelty of this study.

The following are the comments to improve the quality of the manuscript.

Major comments:

1. The Introduction part is looking very general like in many other published papers. There is no strong rationale to screen another strain that produced very low levels of L-asparaginase.

2. It was mentioned in ASNase assay that 1.5 μl of Trichloro Acetic Acid 1.5 M was added to stop the reaction. Eager to know how this lower volume **1.5 μl** TCA will stop the reaction and can be accurate? It is better to include the equation to calculate the activity.

3. The results of this study in terms of activity measured is very low (8.5 U/ml to 15 U/ml) compared to other commercial L-asparaginases (*E. coli*) and other novel reported sources. Moreover, the study is titled the enzyme is 'glutaminase free L-asparaginase'; however, no evident experiments or data reported in this study for claiming this.

4. Did the authors also check for urease activity?

5. Tables are numbered 1-5, showing only coding and interpretation results obtained from Taguchi statistical analysis. Many can be compiled as a single table and if necessary can be filed as supplementary data. For figure no.3, more description needed and clarify the 2 curves shown in each plot.

6. What is the rationale in choosing only four variables for optimization? Why did the authors screen the variables initially?

7. Did the authors try to fit the experimental results to any quadratic equation?

8. How did the Taguchi experimental design optimize the four variables? How did authors arrive at the optimal values and verify?

9. At optimal settings, the expected result is ~ 19 U/ml but the authors obtained only 8.51 U/Ml. The huge variation between experimental and predicted values suggests that the design is not able to perform to good optimization.

10. There is no growth data provided in the manuscript. The experimental values represented in Table 1 corresponds to what time of growth?

11. The study showed mangrove *Rhizophora mucronata* as producers of L-asparaginase and optimization of its production parameters. More experiments must be included to reveal its biochemical properties, glutaminase free activity, molecular weight, and purity to explore it into application level as mentioned in the introduction part.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Industrial Biotechnology, Bioprocess Engineering, Fermentation, Enzymology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 May 2020

Asep Awaludin Prihanto, Faculty of Fisheries and Marine Science, Brawijaya University, Malang, Indonesia

1. The Introduction part is looking very general like in many other published papers. There is no strong rationale to screen another strain that produced very low levels of L-asparaginase.
The reason for the need for exploring the new source is added to the first sentenced in the last paragraph.

2. It was mentioned in ASNase assay that 1.5 µl of Trichloro Acetic Acid 1.5 M was added to stop the reaction. Eager to know how this lower volume **1.5 µl** TCA will stop the reaction and can be accurate? It is better to include the equation to calculate the activity.
We have miswritten the amount of the TCA, The TCA volume was revised.
Formula for calculating the enzyme activity was added to the manuscript

3. The results of this study in terms of activity measured is very low (8.5 U/ml to 15 U/ml) compared to other commercial L-asparaginases (*E. coli*) and other novel reported sources. Moreover, the study is titled the enzyme is 'glutaminase free L-asparaginase'; however, no evident experiments or data reported in this study for claiming this.
Several studies for endophytic bacteria showed lower production than these bacteria. For example the report from Hatamzadeh et al. (2020), PeerJ, DOI10.7717/peerj.8309.
The glutaminase free-L-asparaginase activity was confirmed in our preliminary experiment when investigated the activity onto M9 modified agar supplemented L-Asparagine and L-Glutamine. In the L-Glutamine –supplemented M9 media, the bacteria don't show the activity (the color of media remains yellow). Meanwhile, Asparagine –supplemented M9 media turn blue. We also confirmed the nil activity of the cell-free supernatant.

4. Did the authors also check for urease activity?
No, we did not check the Urease activity

5. Tables are numbered 1-5, showing only coding and interpretation results obtained from Taguchi statistical analysis. Many can be compiled as a single table and if necessary can be filed as supplementary data. For figure no.3, more description needed and clarify the 2 curves shown in each plot.
We afraid if we compiled the Tables, then the clarity and the flowing of the manuscript will also difficult to be followed. Hence, we prefer to show the detail on the tables as in the manuscript. The additional description of the manuscript was added.

6. What is the rationale in choosing only four variables for optimization? Why did the authors screen the variables initially?
We have performed the initial investigation toward five individual factors namely, temperature, L-ASN, pH, inoculum size, and shaker speed. In our preliminary investigation, shaker speed failed to give a significant effect on the production of

L-ASNase. Hence, we only applied four variables (L-ASN, temp, pH, and inoculum size).

7. Did the authors try to fit the experimental results to any quadratic equation?
No, we did not, in the Taguchi method we automatically are recommended the best equation /formula in order to obtain the best production.

8. How did the Taguchi experimental design optimize the four variables? How did authors arrive at the optimal values and verify?
By using Qualitek-4 software for Taguchi experimental, we directly we are automatically directed with the formulation for the production of the most optimum enzymes accompanied by an estimate of its production (Table 5.)

9. At optimal settings, the expected result is ~ 19 U/ml but the authors obtained only 8.51 U/Ml. The huge variation between experimental and predicted values suggests that the design is not able to perform to good optimization.
It is true that the result of the estimated production is higher than that of the exact production. However many researchers also experienced a similar result, where the exact value is lower than the predicted one. As examples is reported by Chenthamarakshan, A., Parambayil, N., Miziriya, N. *et al.* Optimization of laccase production from *Marasmiellus palmivorus* LA1 by Taguchi method of Design of experiments. *BMC Biotechnol* 17, 12 (2017). https://doi.org/10.1186/s12896-017-0333-x; and Shehata AN, Abd El Aty AA. 2014. Optimization of Process Parameters by Statistical Experimental Designs for the Production of Naringinase Enzyme by Marine Fungi. International Journal of Chemical Engineering. ID 273523. https://doi.org/10.1155/2014/273523. The main focus is on the increase in production by comparing before and after optimization.
In the optimized formulation, we reached the increase of the production by three folds. Hence, we believe that the optimization worked.

10. There is no growth data provided in the manuscript. The experimental values represented in Table 1 corresponds to what time of growth?
The culture time is 24 h. we have revised the manuscript to clarify the time of harvesting

11. The study showed mangrove *Rhizophora mucronata* as producers of L-asparaginase and optimization of its production parameters. More experiments must be included to reveal its biochemical properties, glutaminase free activity, molecular weight, and purity to explore it into application level as mentioned in the introduction part.
We have confirmed the glutaminase free activity by investigating using L-glutamine as a substrate. The further characteristic has been under investigation. Next paper will discuss the purification and characteristic of the enzyme from *Lysinibacillus fusiformis* B27

Competing Interests: No competing interests were disclosed.

Reviewer Report 18 December 2019

https://doi.org/10.5256/f1000research.23313.r56818

? **Evans Manyara Nyakeri** iD

Africa Centre of Excellence in Sustainable Use of Edible Insects as Food and Feeds (INSEFOODS) and School of Agricultural and Food Sciences, Jaramogi Oginga Odinga University of Science and Technology, Bondo, Kenya

The study topic is important as it clearly illustrates the need for optimising the production of the enzyme L-ASNASE.

I however have a few suggestions that can contribute towards improving the work:

1. The authors should consider revision of the topic to capture the gist of the study which isolation endophytic bacteria that produce the enzyme glutaminase free L-ASNASE mangrove forest. Suggestion: Isolation of L-ASNASE producing endophytic bacteria and optimization of production of the enzyme. The topic as it is suggest that the study was targeting the isolation of the *Lysinibacillus fusiformis* B27 yet this was the finding of the study rather than the intentional target as from the start.

2. The study is not clearly bringing out the problem of the study: For example: the study rightly points out the limitations of L-ASNAS in paragagh 2 and 3. However, it does not point out the source of L-ASNAS with these limitations and the reason for advocating for alternative sources such as the endophytic bacteria from the mangrooves. Furthermore, the reason given for advocating the sourcing from microbes is not consistent in solving the prior identified limitations of the enzyme: the rapid multiplication and easy management of microbes vis avis the sensitivity to high temperatures (ie. need for extremophilic enzyme).

3. The study has not clearly stated the relationship between substrate glutamine and the enzyme L-ASNase and the need for microbes able to produce asparaginase and not glutaminase nor both. This needs to be established in the introduction. Is it because the two enzymes are antagonistic? The idea was to screen for bacteria isolates capable of producing only the enzyme asparaginase and not glutaminase or both gluataminase and asparaginase., Why discriminate on glutaminase? What is the basis for this? This has not been explained. Is is because the two enzymes are antagonistic?

4. Again, how did you identify isolates that were producing the enzymes? Formation of clearance zones, testing of media for presence of substrates and substrate level reduction, change of media colour especially due to bromothymol blue indicator? Explain to make this clearer.

5. The methodology on screening of enzyme producing bacterial isolates is not conclusively described. Suggestion: state that the plating of the bacteria on the two media types was done by replica plating.

6. The molecular study is adequate in methodology, results and interpretation.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microbiology, biotechnology, food security and applied insect science.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Dec 2019

Asep Awaludin Prihanto, Faculty of Fisheries and Marine Science, Brawijaya University, Malang, Indonesia

I, however, have a few suggestions that can contribute towards improving the work:

1. The authors should consider revision of the topic to capture the gist of the study which isolation endophytic bacteria that produce the enzyme glutaminase free L-ASNASE mangrove forest. Suggestion: Isolation of L-ASNASE producing endophytic bacteria and optimization of production of the enzyme. The topic as it is suggest that the study was targeting the isolation of the *Lysinibacillus fusiformis* B27 yet this was the finding of the study rather than the intentional target as from the start.

-->As suggested by the reviewer, we revised the title to "**Isolation of glutaminase-free L-asparaginase producing endophytic bacteria and optimization of production of the enzyme**"

2. The study is not clearly bringing out the problem of the study: For example: the study rightly points out the limitations of L-ASNAS in paragraphs 2 and 3. However, it does not point out the source of L-ASNAS with these limitations and the reason for advocating for alternative sources such as the endophytic bacteria from the mangroves. Furthermore, the reason given for advocating the sourcing from microbes is not consistent in solving the prior identified limitations of the enzyme: the rapid multiplication and easy management of microbes vis avis the sensitivity to high

temperatures (ie. need for extremophilic enzyme).

-->We added one sentence before the end of paragraph 3 with "**.......The drawbacks of the enzyme applications might be overcome by isolating the enzyme from a unique habitats, such as an endophytic bacteria from mangrove. Therefore, new L-ASNase producers need to be explored.**

3. The study has not clearly stated the relationship between substrate glutamine and the enzyme L-ASNase and the need for microbes able to produce asparaginase and not glutaminase nor both. This needs to be established in the introduction. Is it because the two enzymes are antagonistic? The idea was to screen for bacterial isolates capable of producing only the enzyme asparaginase and not glutaminase or both gluataminase and asparaginase., Why discriminate on glutaminase? What is the basis for this? This has not been explained. It is because the two enzymes are antagonistic?

-->We have revised the third sentence on paragraph 3 with"**....In medical applications, it has a low half-life, allergic reactions, and unnecessary glutamine hydrolysis on L-asparaginase due to the lack of *asparagine synthetase* in cancer cells.**

4. Again, how did you identify isolates that were producing the enzymes? Formation of clearance zones, testing of media for the presence of substrates and substrate-level reduction, change of media colour especially due to bromothymol blue indicator? Explain to make this clearer.

-->We have revised the paragraph on Section of Result and Discussion, subsection Screening and Identification with "**The screening results showed that 31 endophytic mangrove isolates could produce the L-ASNase enzyme shown by blue color-formed surrounding colony (Figure 1A). The change of the color due to the increase of pH resulting from enzyme activity. The best enzyme producer was isolate B27. This isolate had a more extensive and darker blue tone compared to other isolates. Furthermore, only isolate B27 produced glutaminase free-L-asparaginase. Hence, further molecular identification was applied only to the B27 isolate. Further, Gram stain analysis revealed that the bacterium was Gram-positive (Figure 1B)**.

5. The methodology on screening of enzyme-producing bacterial isolates is not conclusively described. Suggestion: state that the plating of the bacteria on the two media types was done by replica plating.

-->We have revised on Section Methods, subsection Isolation and screening of L-ASNase producers in paragraph two, sentence six with "All isolates were streaked onto two different media (M9 medium with L-ASN, and M9 medium with L-glutamine) in duplicates. The plates were incubated at 37°C overnight.

6. The molecular study is adequate in methodology, results and interpretation.

-->Thank you for your comment

Competing Interests: We declare there is no competing interest

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