Electronic Journal of Biotechnology 71 (2024) 10–18

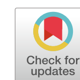
Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

Research article

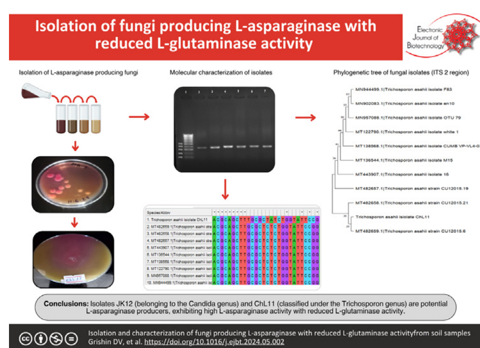
Isolation and characterization of fungi producing L-asparaginase with reduced L-glutaminase activity from soil samples ☆



Tekeba Sisay [a,*], Victor Atunga Mobegi [b], Sabina Wachira [c], Naomi Maina [a,d]

[a] Department of Molecular Biology & Biotechnology, Pan African University Institute for Basic Sciences, Technology and Innovations (PAUSTI) Nairobi, Kenya
[b] Department of Biochemistry, Faculty of Science and Technology, University of Nairobi, Nairobi, Kenya
[c] Kenya Medical Research Institute, Center for Traditional Medicine & Drug Research, Nairobi, Kenya
[d] Biochemistry Department, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

GRAPHICAL ABSTRACT



Isolation of fungi producing L-asparaginase with reduced L-glutaminase activity.

ARTICLE INFO

Article history:
Received 3 January 2024
Accepted 6 May 2024
Available online 24 June 2024

Keywords:
Acute lymphoblastic leukemia
Candida
Fungi
L-asparaginase
Reduced L-glutaminase activity
Soil simples
Trichosporon

ABSTRACT

Background: L-asparaginase (L-ASNase) is an essential enzyme used to treat acute lymphoblastic leukemia (ALL) by depleting L-asparagine, a vital nutrient for leukemia cells. However, its clinical use is challenged by adverse effects linked to its bacterial origin and L-glutaminase (L-GLNase) co-activity. This study aims to identify fungi capable of producing L-ASNase with reduced L-GLNase co-activity.
Results: Among the fungal iolates, isolate JK12 and ChL11 showed high L-ASNase activity (34.04 ± 1.83[a] U/ml and 30.84 ± 0.53[b] U/ml, respectively) with reduced L-GLNase co-activity (4.95 ± 0.28[c] U/ml and 4.80 ± 0.02[d] U/ml, respectively). Sequencing of the internal transcribed spacer (ITS) region of these isolates identified them as *Candida palmioleophila isolate JK12* (≥99% identity with Candida genus) and *Trichosporon asahii isolate ChL11* (≥98% identity with Trichosporon genus). Moreover, these isolates exhibited distinct preferences for carbon (C) and nitrogen (N) sources, as well as culture conditions for L-ASNase production. *C. palmioleophila isolate JK12* demonstrated the highest L-ASNase production in fructose and yeast extract (67.6 ± 0.04[a] U/ml and 51.4 ± 0.04[a] U/ml, respectively), following 96 h of incubation at 25°C (43.8 ± 1.22[a] U/ml, 55.8 ± 0.02[a] U/ml, respectively), with an agitation speed of 100 rpm (59.9 ± 0.04[a] U/ml). On the other hand, *T. asahii isolate ChL11* exhibited maximum L-ASNase production in sucrose and L-asparagine (64.2 ± 0.08[a] U/ml and 63.6 ± 0.01[a] U/ml, respectively), after 120 h of incubation at 35°C.

☆ Audio abstract available in Supplementary material.
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso
* Corresponding author.
E-mail address: 21sisay@gmail.com (T. Sisay).

https://doi.org/10.1016/j.ejbt.2024.05.002
0717-3458/© 2024 The Authors. Published by Elsevier Inc. on behalf of Pontificia Universidad Católica de Valparaíso.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Conclusions: The fungal isolates *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12* have been identified as promising L-ASNase sources of safer therapeutic prospects in cancer therapy due to the reduced GLNase co-activity.

How to cite: Sisay T, Mobegi VA, Wachira S, et al. Isolation and characterization of fungi producing L-asparaginase with reduced L-glutaminase activity from soil samples. Electron J Biotechnol 2024. https://doi.org/10.1016/j.ejbt.2024.05.002.

© 2024 The Authors. Published by Elsevier Inc. on behalf of Pontificia Universidad Católica de Valparaíso. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

L-asparaginase (EC 3.5.1.1), also known as L-asparagine amino-hydrolase, is a valuable therapeutic enzyme that is used to treat acute lymphoblastic leukemia (ALL) [1]. It converts L-asparagine (L-Asn) into L-aspartic acid and ammonia leading to the depletion of L-Asn in the bloodstream [2]. This results in an anti-proliferative effect on leukemia cells since they are reliant on extracellular L-Asn due to their inability to produce sufficient amounts of L-Asn [3,4,5]. This dependency creates an opportunity for L-ASNase therapy to selectively target leukemic cells while preserving healthy ones.

L-ASNases can be produced by animals, plants, and various microorganisms, including bacteria, fungi, algae, yeast, and actinomycetes [6,7]. Nonetheless, the extraction processes of L-ASNase from plant and animal sources pose challenges, resulting in insufficient quantities for application [3,8]. On the other hand, due to their ease of cultivation, ability to produce substantial quantities, and suitability for large-scale production and purification, microorganisms, notably *Escherichia coli, Erwinia carotovora*, and *Erwinia chrysanthemi*, are the primary sources of clinical L-ASNases [3,9,10]. Nonetheless, it is crucial to recognize that bacterial-derived enzymes come with a range of adverse effects, including thrombotic events, dyslipidemia, hyperglycemia, neutropenia, and pancreatitis [11,12,13]. According to reports, about 30–40% of patients develop hypersensitivity to bacterial L-ASNase which makes the therapeutic use of L-ASNase for leukemia patients a matter of discussion for several years [14,15,16]. Investigations indicate that the adverse effects of bacterial L-ASNase are caused by the allergenic prokaryotic epitopes and its ability to hydrolyze L-glutamine [17,18,19]. It has been indicated that L-ASNase with high L-GLNase activity has more severe adverse effects than L-ASNase with lower L-GLNase activity [16,20].

The severe adverse effects associated with bacterial L-ASNases due to the prokaryotic allergenic epitopes initiate investigations to find new sources that produce L-ASNase with minimal side effects [18]. Concerning this, L-ASNase derived from eukaryotes is attracting attention as it has been reported to have lower adverse effects [21]. Fungi have attracted attention for their potential in L-ASNase production due to their eukaryotic nature and cellular resemblance to humans [22,23]. It is anticipated that fungal L-ASNases would demonstrate higher similarity to mammalian proteins compared to prokaryotic ones, potentially resulting in reduced immunological reactions [24].

2. Materials and methods

2.1. Sample collection and isolation of fungi

Thirty-six soil samples (nine from each sampling site), weighing one hundred grams each, were collected at a depth of 0 to 30 cm from four locations across the Republic of Kenya. These locations include the Jomo Kenyatta University of Agriculture and Technology campus (1.0912° S, 37.0117° E), family poultry waste disposal sites near Juja town market (1.1002° S, 37.0183° E), Karura forest (1.2368° S, 36.8304° E), and Thika town waste dumping site (1.0388° S, 37.0834° E). The collection was conducted using sterile bottles and a sterile spatula at each site, and the samples were stored at 4°C until used.

In the present study, fungal strains were isolated from serially diluted soil samples (dilution factor of 10^{-3}) using the dilution plate method on Modified Czapek Dox (MCD) agar, consisting of glucose (0.2%), L-Asn (1%), agar (2%), $MgSO_4 \cdot 7H2O$ (0.052%), KCl (0.052%), KH_2PO_4 (0.152%), $CuNO_3 \cdot 3H_2O$ (0.001%), $FeSO_4$ (0.001%), $ZnSO_4 \cdot 7H_2O$ (0.001%), and Phenol red (0.005%, v/v) at a pH of 6.6 [25,26,27]. The isolates exhibiting a pink halo zone around fungal growth were identified as L-ASNase producers and sub-cultured for further study.

2.2. Screening of isolates for L-asparagine and L-glutamine hydrolytic enzyme

The isolated fungal strains were screened for their potential to produce L-ASNase enzyme without L-GLNase activity. For this purpose, the isolates were assessed for their ability to utilize L-glutamine by cultivating them on MCD media with L-glutamine as the sole nitrogen source [28]. Then, they were also screened for their capacity to secrete L-ASNase growing them on MCD media supplemented with L-Asn as the sole nitrogen source and 0.005% phenol red [25]. The secretion of L-ASNase by the isolates was indicated by the formation of a pink zone due to the hydrolysis of L-Asn, and the production of ammonia. The strains exhibiting the highest enzyme activity zone index value were selected for further investigation. The enzyme activity halo zone index was calculated as follows [27]:

$$\text{Halo zone Index} = \frac{\text{Halo Zone diameter (cm)}}{\text{Colony diameter (cm)}}$$

Where the halo zone diameter is measured in cm and refers to the size of the pink-colored region formed around the growing mycelia, whereas the colony diameter, also measured in cm, represents the size of the growing mycelia created at the center of the halo zone.

2.3. Determination of fungal extracts L-asparaginase and L-glutaminase activity

The specificity of L-ASNase for L-Asn and L-glutamine was evaluated according to the Nesslerization method with some modifications [29]. The crude enzyme was extracted from the MCD broth after 72 h incubation by centrifugation and a supernatant, containing enzyme, was used to determine the enzymatic activity of the extract using the respective substrates, L-Asn for L-ASNase activity and L-glutamine for L-GLNase activity. In both cases, the enzymatic activity of the extract was determined by mixing 1 ml of 50 mM Tris-HCl buffer (pH 8.4), 0.1 ml of 189 mM L-Asn or L-glutamine, 0.9 ml of distilled water, and 0.1 ml of crude enzyme, shaken well and incubated at 37°C for 30 min. Then, the reaction was terminated by the addition of 0.1 ml of 1.5 M trichloroacetic acid. After centrifuging the mixture at 12,000 rpm for 15 min, 0.2 ml supernatant was taken and mixed with 2 ml of distilled water and

0.2 ml Nessler's reagent. Then, the ammonia liberated from the reaction was quantified by measuring the absorbance of the mixture at 436 nm with a UV-spectrophotometer after drawing a standard curve using Ammonium sulfate as a standard solution. One international unit (IU) of L-ASNase activity is defined as the amount of enzyme required to produce 1 μmol of ammonia per minute under the conditions of the assay [29]. The enzymatic activity was calculated as follows:

$$\text{Enzyme activity}(\text{U/mL}) = \frac{(\mu\text{mole of ammonia liberated})(2.20)}{(0.2)(30)(0.1)}$$

Where, 2.20 = Final volume of reaction system, 0.2 = Volume taken for OD, 0.1 = Enzyme aliquots, 30 = Incubation time (in min).

2.4. Identification of L-ASNase-producing isolates

For molecular characterization of the selected fungi exhibiting notable extracellular hydrolytic enzyme production, DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (ver.2.2.2), following the kit instructions; the concentration and purity of the DNA extract were determined with a NanoDrop spectrophotometer. Subsequently, the ITS-2 region of the extracted DNA was amplified using universal primers ITS3 and ITS4 [30] in a 50 μL reaction mixture consisting of 3 μL DNA (100 ng), 1.0 μl of forward and reverse primers each (50 μM), 25 μL 1X Master Mix, and 20 μL nuclease-free water. The amplification process was initiated by pre-heating at 95°C for 10 min, followed by 34 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Subsequently, the PCR amplicons with a length of 350 bp were forwarded to Macrogen (Netherlands) for sequencing.

The sequences obtained from Macrogen were compared with the GenBank database with the help of the BLASTn program (https://www.ncbi.nlm.nih.gov/BLAST). With consideration of nucleotide similarity percentage, the best ten BLAST hits were selected for each isolate and used to construct a midpoint-rooted phylogenetic tree by neighbor-joining phylogenetic tree using the neighbor-joining method on MEGA 11 software [31]. One thousand bootstrap replications were calculated to evaluate the tree topology [32]. The sequences of the isolates were then submitted to the GenBank database, and the respective GenBank accession numbers were acquired.

2.5. Optimization of L-ASNase production media components and culture conditions

L-ASNase production conditions, such as incubation temperature, initial pH of the media, fermentation period, inoculum volume, and agitation speed; and production media (MCD) components, such as carbon and nitrogen sources were optimized. The optimization process began with the refreshment of the isolates for 72 h in MCD containing 10% (v/v) L-Asn. Then, the inocula were used to optimize a range of conditions, including incubation temperature (ranging from 20-40°C), initial pH (varying from 5.0 to 9.0) of MCD media, fermentation period (ranging from 48-120 h), agitation speeds (ranging from 0 rpm-200 rpm), and inoculum volume (1–16%). These fungal culture conditions are pivotal factors that significantly impact enzyme production due to their direct effect on the regulation of protein expression, which in turn changes the composition of the transcriptome.

Furthermore, the study investigated the efficacy of common carbon sources (such as glucose, sucrose, fructose, sodium acetate, and starch) and nitrogen sources (including L-Asn, L-glycine, yeast extract, $NaNO_3$, and peptone), which serve as primary components in many L-ASNase production media. The optimum conditions were evaluated in terms of the enzyme activity produced using the standard method [29].

2.6. Statistical analysis

All statistical analyses of the experimental data were carried out using Statistical Package for Social Sciences Software (SPSS) version 20. All descriptive statistical analyses were presented as mean ± SD of triplicate samples using SPSS version 20. Moreover, SPSS.20 was used for Tukey's honestly significant difference (HSD) test at a significance level of p = 0.05 to compare means using one-way ANOVA. The statistical graph (Fig. 2) was created using MS Excel 2010.

3. Results and discussion

3.1. Isolation and screening of L-asparaginase-producing fungal

In the present study, sixty-five fungal isolates were isolated based on the halo zone formation around fungal mycelia growing on MCD culture media that contains L-Asn as the sole nitrogen source. Among these isolates, seventeen of them showed a wide range of L-ASNase production capabilities with low L-glutamine utilization capacity as shown in Fig. 1. The formation of a halo zone on the medium, resulting from the shift in color from yellow to pink, indicates the rise of the culture media pH due to the liberation of ammonia molecules from L-Asn by the L-ASNase activity of the isolates [3]. It is also important to mention that ammonia can be liberated from the breakdown of L-glutamine by the L-GLNase co-activity of L-ASNase even though L-ASNase with L-GLNase co-activity is an unsafe enzyme for treating ALL patients [28,33].

Statistical analysis of the L-ASNase activity halo zone index mean indicated significant differences among different isolates, as illustrated in Fig. 2. The isolates ChL11 and KA17 showed the

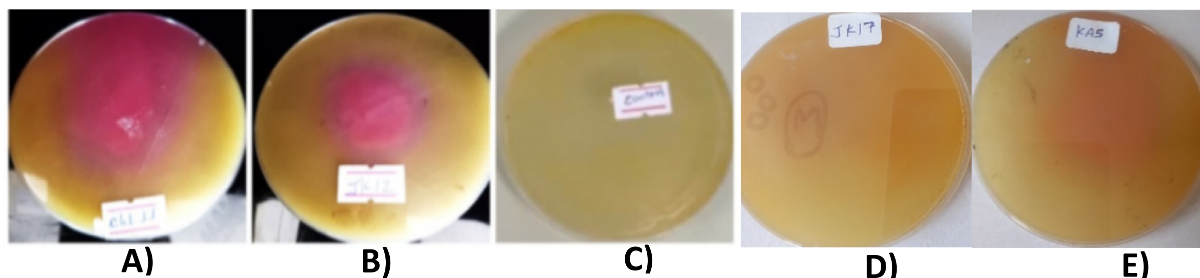


Fig. 1. Plate assay showing halo zone formed due to L-asparagine hydrolysis by L-asparaginase-producing fungal isolates. Fungal isolates ChL11 (A) and JK12 (B) exhibit L-ASNase activity, releasing ammonia from L-Asn in MCD and causing a color change from yellow (C, control plate) to pink. Fungal isolates JK17 (D) and KA5 (E) display L-ASNase with L-GLNase co-activity, releasing ammonia from L-glutamine in MCD and inducing a slight color change from yellow (C, control plate) to pink after 72 h of incubation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

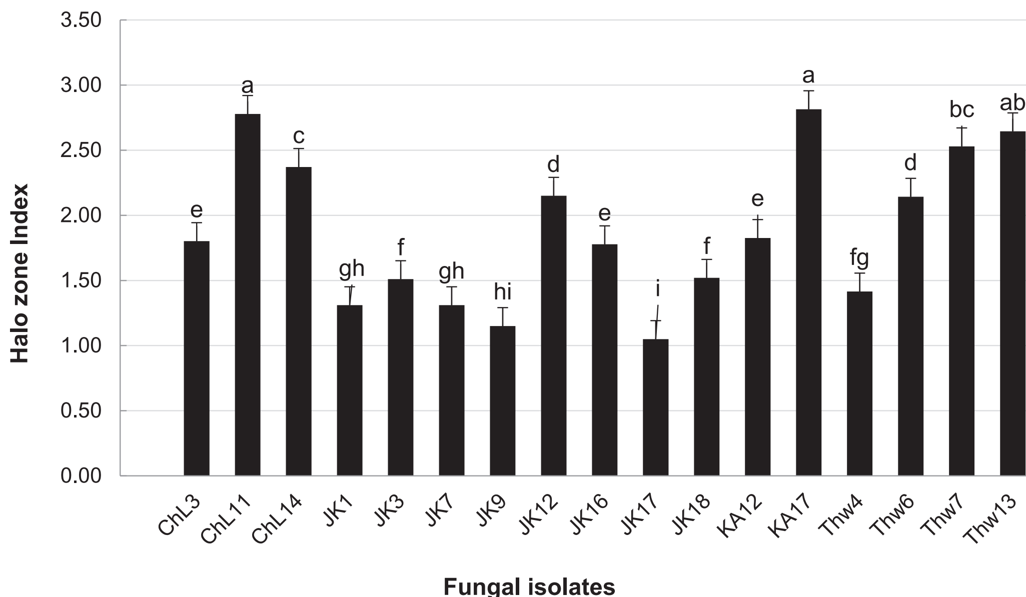


Fig. 2. Bar graph showing fungal isolates' L- ASNase activity halo zone index after 72 h incubation on MCD. Different lowercase letters on the top of the bars indicate significant differences at the 5% level (Tukey's HSD test).

highest mean L-ASNase activity halo zone index, while isolates ThW7, ThW13, ChL14, Thw6, and JK12 demonstrated a mean L-ASNase activity halo zone index significantly higher than the mean observed for the remaining isolates. These isolates, ChL11, KA17, ThW7, ThW13, ChL14, Thw 6, and JK12, displayed an L- ASNase activity halo zone diameter that was twice or more than twice the size of the colony diameter (index ≥ 2) which is in line with the discovery reported by Doriya and Kumar [28]. This finding suggests that these isolates are potential L-ASNase producers, aligning with the observation that a wider halo zone formation around the mycelia indicates a greater capacity to utilize L-Asn due to L-ASNase production [34,35].

3.2. Determination of fungal extracts L-asparaginase and L-glutaminase activity

In the present study, the L-ASNase extracted from the isolate JK12 demonstrated the highest mean L-ASNase activity (34.04 ± 1.83[a] U/ml) followed by L-ASNase extracted from ChL11(30.84 ± 0.53[b] U/ml). In addition to exhibiting the maximum L-ASNase activity, the enzyme from these isolates showed lower GLNase activity (4.80 ± 0.02[d] U/ml, 4 and, 4.95 ± 0.28[c] U/ml, respectively) compared with the extract from the isolates Thw7, Thw13, and Thw6 (5.43 ± 0.26[b] U/ml, 5.45 ± 0.05[b] U/ml and 5.59 ± 0.32[a] U/ml, respectively).

On the other hand, while the enzyme extracted from isolates KA17 and ChL14 exhibited comparable lower GLNase activity (4.82 ± 0.12[d] U/ml and 4.94 ± 1.72[c] U/ml) to that of ChL11 and JK12, their L-ASNase activity (29.52 ± 2.75[d] U/ml and 23.26 ± 4.22[g] U/ml) is significantly lower than that of ChL11 and JK12 (30.84 ± 0.53[b] U/ml and 34.04 ± 1.83[a] U/ml, respectively). In consideration of the extracts' L-ASNase and GLNase activity, the extract from JK12 showed a higher L-ASNase to GLNase activity ratio (6.88) followed by the extract from isolate ChL11 (6.43) as depicted in Table 1. This finding indicates that the extracts from these isolates, exhibiting substantially higher L-ASNase activity (more than sixfold) compared to their GLNase activity, have the potential to produce L-ASNase with minimal side effects [20]. Although the fungal isolates exhibited enzymes with lower GLNase activity compared to their L-ASNase activity, it is worth noting that the L-ASNases extracted from all the isolates still demonstrated GLNase co-activity.

3.3. Molecular characterization of fungal isolates

The amplification of the ITS-2 region of genomic DNA and agarose gel electrophoresis confirmed that the size of the PCR products

Table 1
Determination of the fungal isolates' extract L-ASNase and L-GLNase activity.

No.	Isolates	**L-ASNase activity (U/ml)**	**L-GLNase activity (U/ml)**	**L-ASNase activity to L-GLNase activity ratio**
1	ChL11	30.84 ± 0.53[b]	4.80 ± 0.02[d]	6.43
2	ChL14	23.26 ± 4.22[g]	4.94 ± 1.72[c]	4.71
3	JK12	34.04 ± 1.83[a]	4.95 ± 0.28[c]	6.88
4	KA17	29.52 ± 2.75[d]	4.82 ± 0.12[d]	6.12
5	Thw6	28.68 ± 2.06[e]	5.59 ± 0.32[a]	5.13
6	Thw7	29.87 ± 2.06[c]	5.43 ± 0.26[b]	5.50
7	Thw13	25.86 ± 2.28[f]	5.45 ± 0.05[b]	4.74

Means within the same column followed by different letters are statistically different $p \leq 0.05$ (Tukey's HSD test).

for the selected isolates is approximately 350 bp (Fig. 3), consistent with the findings reported by Kumar and Shukla [36]. Conversely, White et al. [37] have reported that the amplicon size of fungal rRNA using ITS3 and ITS4 primers is 330 base pairs.

The analysis of nucleotide homology in the ITS sequences of the isolates using BLAST revealed that ChL11 is classified within the genus Trichosporon. The Trichosporon species most closely related to isolate ChL11 include *Trichosporon asahii strains CU12015.6*, *CU12015.21*, and *CU12015.19*, along with isolates *16, M15*, *CUMB VP-VL4-03*, *white 1*, *OTU_79, F83*, and *en10* (Fig. 4A), sharing a minimum nucleotide identity of 98.0%. The fungal isolate identified in this study, ChL11, is classified as *T. asahii isolate ChL11*, with its assigned GenBank accession number being OR770496, aligning with the findings of Ashok et al. [12], who identified *T. asahii IBBLA1* as an L-ASNase producer. In addition, Ashok and Kumar [38] reported an ASNase enzymatic activity of 20.57 U mL^{-1} after optimizing the physical and chemical parameters.

On the contrary, the ITS-2 sequence analysis revealed that isolate JK12 belongs to the genus Candida. Specifically, fungal species closely related (≥99.0% identity) to isolate JK12 include *Candida palmioleophila isolate 2Y25* (*C. palmioleophila isolate 2Y25*), *C. palmioleophila strain SM94181*, *C. palmioleophila isolate NWHC 44797–149 1SD*, *C. palmioleophila isolate 17H*, *C. palmioleophila isolates FJAT-31035*, *C. palmioleophila strain CBS 7418*, *C. manassasensis strain UCDFST*, *C. palmioleophila strain YFA124396*, *C. palmioleophila strain YFA124099*, and *C. palmioleophila IFM 54668* (Fig. 4B). The fungal isolate from this study, JK12, is designated as *C. palmioleophila isolate JK12*, with the GenBank accession number OR770502, consistent with the study's findings. This finding aligns with Arima et al.'s [39] research, revealing Candida's potential to produce extracellular L-ASNase without glutamine hydrolysis which is strengthened when Correa et al. [40] confirmed the capacity of *Candida glaebosa L75* to L-ASNase. Sakamoto et al. [41] and Momeni [42] have also reported that certain yeast strains within *Candida utilis* exhibit a notable capability for extracellular L-ASNase production.

While filamentous fungi like Aspergillus, Fusarium, and Penicillium are commonly known as L-ASNase producers, none of the isolates in the present study belonged to these genera. The absence of Aspergillus, Fusarium, and Penicillium isolates in this study may be attributed to both geographical differences, as it marks the first research endeavor of its kind in Kenya and the nature of the sampled environment. It is worth noting that many L-ASNase-producing fungi have been predominantly isolated from aquatic environments, which differs from the terrestrial samples collected in this study. The isolation and characterization of fungal isolates in the present study suggest that various fungal genera have the potential to produce L-ASNase, in addition to the filamentous fungi that are commonly reported.

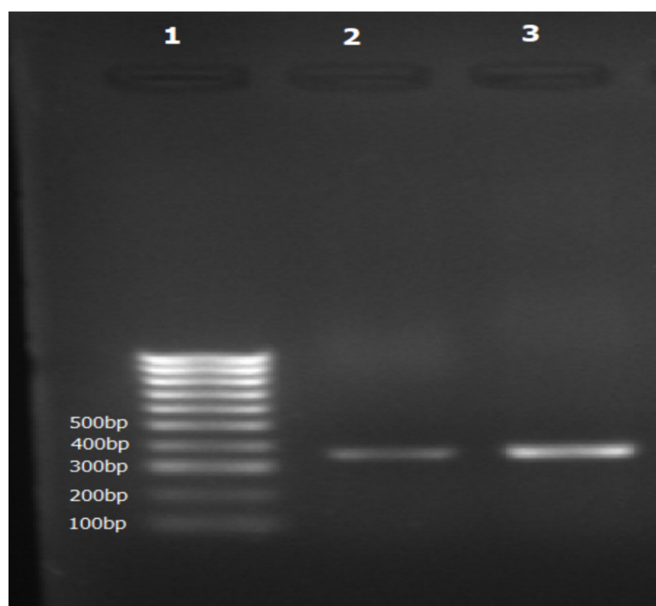


Fig. 3. Illustrates the agarose gel image of PCR products, with Lanes 2, and 3 displaying PCR amplicons of the ITS II region for fungal isolates (ChL11, and JK12), and Lane 1 featuring a 100 bp ladder.

3.4. Optimization of L-ASNase production media component and culture conditions

3.4.1. Effect of carbon and nitrogen sources on L-ASNase production

As carbon and nitrogen sources play a crucial role in the physiology and metabolism of enzyme production by fungi, the effect of carbon and nitrogen sources on L-ASNase production was studied in the present study to explore the preferred source that can induce ASNase enzyme production. The findings in the current study demonstrated significant variations in the quantity of L-ASNase produced by both isolates in an MCD medium with diverse 0.2% carbon sources as illustrated in Table 2. The investigation revealed that among the tested carbon sources, sucrose and fructose exhibited the highest effectiveness, resulting in maximum L-ASNase production for *T. asahii isolate ChL11* (64.2 ± 0.08[a] U/ml) and C. *palmioleophila isolate JK12* (67.6 ± 0.04[a] U/ml), respectively. These results align with the findings of Doriya and Kumar [28], who found that sucrose and fructose were effective in supporting L-ASNase production, while glucose served as a good inducer and primary carbon source for L-ASNase biosynthesis. Moreover, Moguel et al. [43] have also found that the production of L-ASNase by *Streptomyces albidoflavus* was favored when sucrose was used as a carbon source.

Statistical analysis of the results revealed that the nitrogen sources have a substantial influence on the production of L-ASNase by both *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12*. The highest L-ASNase levels (63.6 ± 0.10[a]) were produced by *T. asahii isolate ChL11* and in an MCD medium containing L-Asn as the sole nitrogen source. On the other hand, the maximum L-ASNase (51.4 ± 0.04[a]) production was observed by *C. palmioleophila isolate JK12* when yeast extract as a nitrogen source, as illustrated in Table 2. A similar finding was reported by Udayan et al. [44] which revealed that endophytic fungus *Fusarium* sp. *LCJ324* showed the highest L-ASNase production when L-Asn was used.

3.4.2. Effect of pH on L-ASNase production

In the present study, the isolates *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12* demonstrated maximum L-ASNase production, reaching 52.0 ± 0.10[a] U/ml in an MCD medium with an initial pH of 8.0 and 57.3 ± 0.26[a] to 59.6 ± 0.00[a] U/ml, in an MCD medium with an initial pH of ranging 6.5 to 7.0, respectively, incubated at 25°C for 72 h (Table 3). The current result aligns with Ashok et al. [12] discovery that *T. asahii* IBBLA1 achieves maximum L-ASNase production at a pH of 7.0 with an incubation period of 60 h. On the other hand, Rajesh et al. [45] have found that a high enzyme activity was obtained at pH 8.0, while L-ASNase was produced by *Aspergillus terreus* for 48 h. Contrary to the present finding, Udayan et al. [44] discovered that the optimal L-ASNase activity was attained at an initial pH of 6.0. These results, indicating the presence of substantial differences in L-ASNase production levels at different pHs both by *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12,* may suggest the influence of the production medium's pH on the microbial production of L-ASNase.

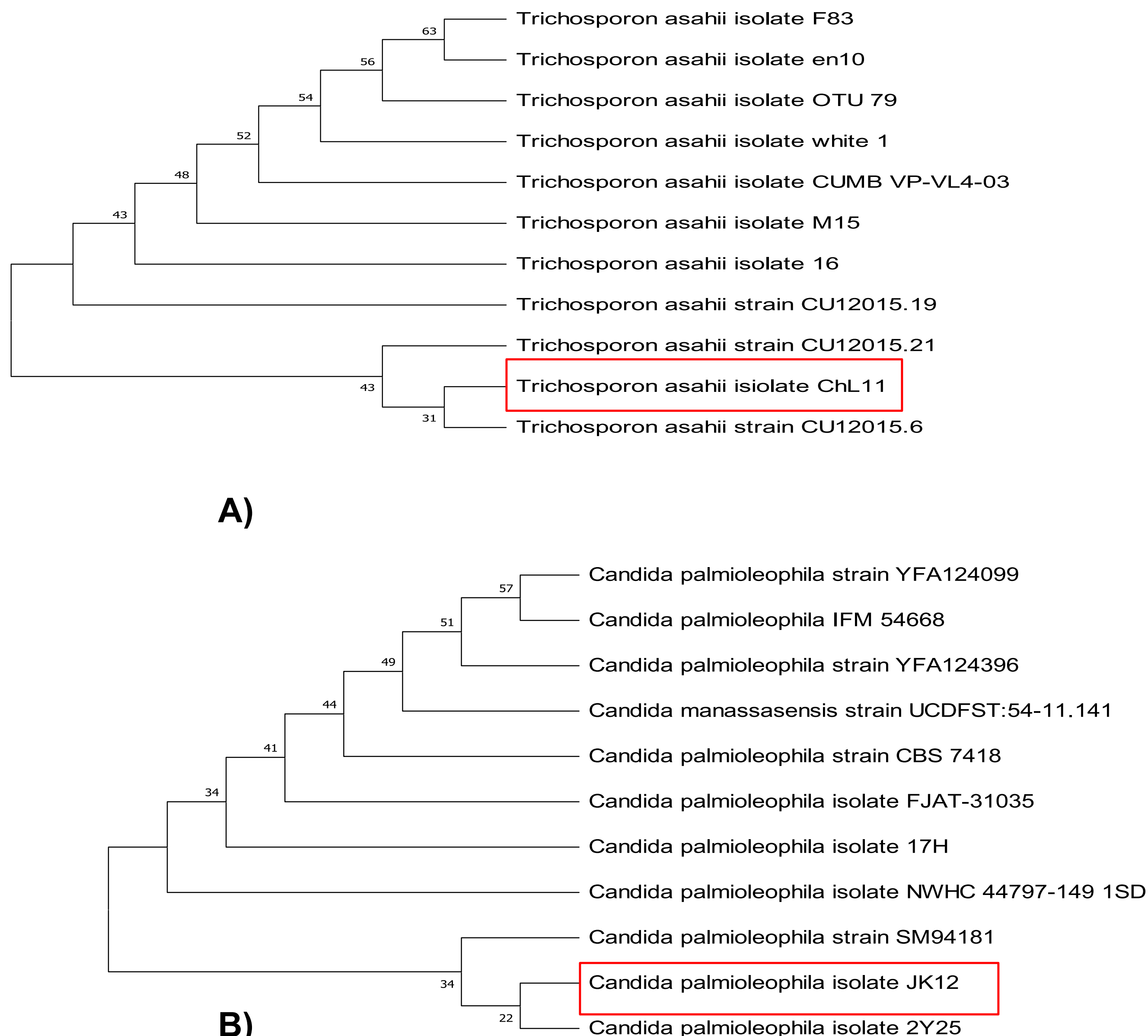


Fig. 4. Depicts the phylogenetic trees of fungal isolates generated through the neighbor-joining method with the Kimura 2-parameter model. Figures A and B present the phylogenetic trees for *isolates T. asahii* (*ChL11*) and *C. palmioleophila* (*JK12*) respectively, along with their corresponding homologous isolates in NCBI. The numbers adjacent to each node represent the percentage bootstrap value based on 1000 replicates. The fungal isolate identified in this study is highlighted within a red box in each tree.

3.4.3. Effect of incubation temperature and period on L-ASNase production

In the present study, the isolate *T. asahii* isolate ChL11 exhibited maximum L-ASNase production (54.7 ± 0.57^a U/ml) at an incubation temperature of 35°C, while *C. palmioleophila isolate JK12* produced a maximum L-ASNase (55.8 ± 0.02^a U/ml) at an incubation temperature of 25°C, incubated for 72 h in an MCD medium (Table 4). Moreover, the isolate *T. asahii isolate ChL11* exhibited a maximum L-ASNase production (65.6 ± 0.01^a U/ml) at an incubation period of 120 h, while *C. palmioleophila isolate JK12* produced a maximum L-ASNase (43.8 ± 1.22^a U/ml) at an incubation period of 96 h at an incubation temperature of 25°C. This discovery in the current study is in line with the report found by Ashok et al. [12] that *T. asahii* IBBLA1 produced L-ASNase optimally at an incubation period of 60 h. These results suggest that the microbial production of L-ASNase may be influenced by the incubation temperature and incubation period. Furthermore, Varalakshmi and Raju [46] observed the significant influence of incubation duration on L-ASNase production, noting that various organisms reach peak L-ASNase production at distinct incubation times, as emphasized by Abo-Stait et al. [47] for various fungal isolates.

3.4.4. Effect of agitation speed and inoculum volume on L-ASNase production

The results in the present study revealed substantial variations in the quantity of L-ASNase produced by both isolates, *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12,* in an MCD medium with different agitation speeds and inoculum volume percentages relative to the production medium volume, as illustrated in Table 5. *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12* exhibited the highest L-ASNase production (57.0 ± 0.06^a U/ml and 59.9 ± 0.04^a U/ml, respectively) at an agitation speed of 50 rpm and 100 rpm, respectively. In a study conducted by Ali et al. [48], an agitation speed of 100 rpm was found to be optimal for L-ASNase production by *Aspergillus sydowii and Fusarium oxysporum* which is similar to the finding recorded for *C. palmioleophila isolate*

Table 2
Effect of carbon and nitrogen sources on L-ASNase production.

Carbon/ Nitrogen source		L-ASNase activity (U/ml) from *T. asahii isolate ChL11*	L-ASNase activity (U/ml) from *C. palmioleophila isolate JK12*
Carbon source	Dextrose	33.3 ± 0.20^{e}	40.5 ± 0.18^{d}
	Fructose	55.5 ± 0.18^{b}	67.6 ± 0.04^{a}
	Sucrose	64.2 ± 0.08^{a}	43.8 ± 0.03^{c}
	Sodium acetate	45.3 ± 0.04^{c}	52.2 ± 0.03^{b}
	Starch	37.2 ± 0.15^{d}	38.4 ± 0.06^{e}
Nitrogen source	L-Asn	63.6 ± 0.10^{a}	48.4 ± 0.27^{b}
	Glycine	30.9 ± 0.04^{e}	47.4 ± 0.14^{c}
	$NaNO_3$	35.4 ± 0.07^{b}	33.1 ± 0.08^{e}
	Peptone	34.1 ± 0.19^{c}	37.2 ± 0. 07^{d}
	Yeast extract	30.7 ± 0.13^{d}	51.4 ± 0.04^{a}

Means within the same column in a group followed by different letters are statistically different $p \leq 0.05$ (Tukey's HSD test).

Table 3
Effect of MCD medium initial pH on L-ASNase production.

MCD medium initial pH	L-ASNase activity (U/ml) from *T. asahii isolate ChL11*	L-ASNase activity (U/ml) from *C. palmioleophila isolate JK12*
pH = 5.0	18.0 ± 0.0^{g}	5.1 ± 0.06^{f}
pH = 5.5	27.3 ± 0.0^{f}	26.9 ± 0.00^{d}
pH = 6.0	28.2 ± 0.07^{e}	45.4 ± 0.36^{b}
pH = 6.5	33.5 ± 0.23^{c}	59.6 ± 0.00^{a}
pH = 7.0	41.4 ± 0.06^{b}	57.3 ± 0.26^{a}
pH = 7.5	41.4 ± 0.0^{b}	44.9 ± 0.0^{b}
pH = 8.0	52.0 ± 0.10^{a}	36.3 ± 1.37^{c}
pH = 8.5	29.5 ± 0.06^{d}	20.8 ± 4.56^{d}
pH = 9.0	8.3 ± 0.10^{h}	13.1 ± 4.16^{e}

Means within the same column in a group followed by different letters are statistically different $p \leq 0.05$ (Tukey's HSD test).

Table 4
Effect of temperature and incubation period on L-asparaginase production.

Incubation temperature/ Incubation period		L-ASNase activity (U/ml) from *T. asahii isolate ChL11*	L-ASNase activity (U/ml) from *C. palmioleophila isolate JK12*
Incubation temperature	20°C	33.1 ± 1.97^{c}	30.7 ± 0.5^{e}
	25°C	36.8 ± 2.48^{bc}	55.8 ± 0.02^{a}
	30°C	44.5 ± 8.44^{ab}	43.9 ± 1.02^{b}
	35°C	54.7 ± 0.57^{a}	41.2 ± 0.01^{c}
	40°C	40.4 ± 0.05^{bc}	35.7 ± 0.07^{d}
Incubation period	48 h	28.7 ± 1.74^{c}	28.1 ± 0.5^{d}
	72 h	32.6 ± 2.70^{c}	36.3 ± 0.55^{c}
	96 h	51.5 ± 0.03^{b}	43.8 ± 1.22^{a}
	120 h	65.6 ± 0.01^{a}	38.5 ± 0.01^{b}

Means within the same column in a group followed by different letters are statistically different $p \leq 0.05$ (Tukey's HSD test).

Table 5
Effect of agitation speed and inoculum volume on L-ASNase production.

Agitation speed (rpm) / Inoculum volume % (v/v)		L-ASNase activity (U/ml) from *T. asahii isolate ChL11*	L-ASNase activity (U/ml) from *C. palmioleophila isolate JK12*
Agitation speed (rpm)	0 rpm	48.2 ± 0.25^{c}	39.3 ± 0.05^{e}
	50 rpm	57.0 ± 0.06^{a}	41.7 ± 0.10^{d}
	100 rpm	52.3 ± 0.17^{b}	45.6 ± 0.03^{c}
	150 rpm	44.4 ± 0.07^{d}	51.4 ± 0.05^{b}
	200 rpm	41.8 ± 0.35^{e}	61.6 ± 0.12^{a}
Inoculum volume % (v/v%)	1%	29.6 ± 0.07^{e}	39.7 ± 0.04^{e}
	2%	61.7 ± 0.10^{a}	50.0 ± 0.34^{d}
	4%	57.9 ± 0.57^{b}	55.3 ± 0.25^{b}
	8%	54.4 ± 0.07^{c}	67.7 ± 0.04^{a}
	16%	45.7 ± 0.02^{d}	53.7 ± 0.18^{c}

Means within the same column in a group followed by different letters are statistically different $p \leq 0.05$ (Tukey's HSD test).

JK12 in the present study. These findings suggest that the agitation rate of the medium during incubation is a crucial factor that can affect enzyme productivity in microbes as it determines the mass transfer of oxygen from gas bubbles in the environment, the level of dissolved oxygen in the medium, and the accessibility of nutrients in the medium [49]. Momeni [42] has also reported that the

production of L-ASNase by *C. utilis* is maximum at the agitation speed of 300 rpm. Contrary to this, Yap et al. [50] have indicated that some fungal species like Fusarium proliferatum can produce the same amount of L-ASNase in agitating conditions regardless of the agitation speed.

In addition to agitation speed, the production of L-ASNase is influenced by the percentage of inoculum volume relative to the production medium volume. The maximum L-ASNase production (61.7 ± 0.10[a] U/ml and 67.7 ± 0.04[a] U/ml, respectively) is observed with an inoculum volume of 2% for *T. asahii isolate ChL11* and 4% for *C. palmioleophila isolate JK12*. In contradiction to the findings in the current study, Zia et al. [51] found that maximal L-ASNase production by *Aspergillus niger* was achieved when an inoculum volume of 5% relative to the production medium volume.

4. Conclusions

L-asparaginase is a crucial therapeutic enzyme vital for treating acute lymphoblastic leukemia by disrupting leukemic cells' reliance on extracellular L-Asn. However, the side effects associated with currently available bacterial-derived L-ASNases highlight the necessity to explore eukaryotic organisms as potential novel L-ASNase producers, particularly those with low L-GLNase co-activity. In this study, fungal isolates *T. asahii isolate ChL11* and *C. palmioleophila isolate JK12* exhibited promising L-ASNase with low glutaminase activity. Therefore, this finding is not only aligned with the existing literature suggesting that fungal L-ASNase can be a key therapeutic agent in the treatment of acute lymphoblastic leukemia but also reaffirms that fungal sources for the production of L-ASNase are a promising area that could be investigated further to address the challenges associated with bacterial-derived L-ASNases, including adverse effects like hypersensitivity reactions. Moreover, further studies are imperative to validate the efficacy and safety of fungal-derived L-ASNases for therapeutic use.

Financial support

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Tekeba Sisay: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Victor Atunga Mobegi:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Conceptualization. **Sabina Wachira:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Conceptualization. **Naomi Maina:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

Acknowledgments

The authors would like to sincerely thank the Pan African University Institute of Basic Sciences, Technology, and Innovation (PAUSTI) for providing the reagents and laboratory facilities essential for this study. We extend special thanks to Mr. Patrick Koech and Ms. Portia Osei-Obeng for their assistance in proofreading the manuscript.

Supplementary material

https://doi.org/10.1016/j.ejbt.2024.05.002.

Data availability

Data will be made available on request.

References

[1] Elshafei AM, Hassan MM, Abouzeid MA, et al. Purification, characterization and antitumor activity of L-asparaginase from *Penicillium brevicompactum* NRC 829. Microbiol Res J 2012;2(3):158–74. https://doi.org/10.9734/BMRJ/2012/1735.
[2] El-Hefnawy MA, Attia M, El-Hofy ME, et al. Optimization production of L-asparaginase by locally isolated filamentous fungi from Egypt. Curr Sci Int 2015;4(3):330–41.
[3] Mahajan RV, Saran S, Saxena RK, et al. A rapid, efficient and sensitive plate assay for detection and screening of l-asparaginase-producing microorganisms. FEMS Microbiol Lett 2013;341(2):122–6. https://doi.org/10.1111/1574-6968.12100. PMid: 23398626.
[4] Asselin BL, Ryan D, Frantz CN, et al. *In vitro* and *in vivo* killing of acute lymphoblastic leukemia cells by L-asparaginase. Cancer Res 1989;49(15):4363–8. PMid: 2743326.
[5] Egler RA, Ahuja SP, Matloub Y. L-asparaginase in the treatment of patients with acute lymphoblastic leukemia. J Pharmacol Pharmacother 2016;7(2):62–71. https://doi.org/10.4103/0976-500X.184769. PMid: 27440950.
[6] Batool T, Makky EA, Jalal M, et al. A comprehensive review on L-asparaginase and its applications. Appl Biochem Biotechnol 2016;178:900–23. https://doi.org/10.1007/s12010-015-1917-3. PMid: 26547852.
[7] Muneer F, Siddique MH, Azeem F, et al. Microbial L-asparaginase: Purification, characterization and applications. Arch Microbiol 2020;202:967–81. https://doi.org/10.1007/s00203-020-01814-1. PMid: 32052094.
[8] Abdelrazek NA, Elkhatib WF, Raafat MM, et al. Diverse origins of microbial L-asparaginases and their current miscellaneous applications. Arch Pharm Sci Ain Shams Univ 2019;3(1):21–36. https://doi.org/10.21608/aps.2019.20220.
[9] Meghavarnam AK, Janakiraman S. A simple and efficient dye-based technique for rapid screening of fungi for L-asparaginase production. J Exp Biol Agric Sci 2015;3(2):123–30.
[10] Chand S, Mahajan RV, Prasad JP, et al. A comprehensive review on microbial L-asparaginase: Bioprocessing, characterization, and industrial applications. Biotechnol Appl Biochem 2020;67(4):619–47. https://doi.org/10.1002/bab.1888. PMid: 31954377.
[11] Bergeron S. Asparaginase toxicities: Identification and management in patients with acute lymphoblastic leukemia. Clin J Oncol Nurs 2017;21(5):E248–59. https://doi.org/10.1188/17.CJON.E248-E259. PMid: 28945721.
[12] Ashok A, Doriya K, Rao JV, et al. Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss. Sci Rep 2019;9(1):1423. https://doi.org/10.1038/s41598-018-38094-1. PMid: 30723240.
[13] Brumano LP, da Silva FV, Costa-Silva TA, et al. Development of L-asparaginase biobetters: Current research status and review of the desirable quality profiles. Front Bioeng Biotechnol 2019;6:212. https://doi.org/10.3389/fbioe.2018.00212. PMid: 30687702.
[14] Tabandeh MR, Aminlari M. Synthesis, physicochemical and immunological properties of oxidized inulin–L-asparaginase bioconjugate. J Biotechnol 2009;141(3–4):189–95. https://doi.org/10.1016/j.jbiotec.2009.03.020. PMid: 19433225.
[15] Beckett A, Gervais D. What makes a good new therapeutic L-asparaginase? World J Microbiol Biotechnol 2019;35(10):152. https://doi.org/10.1007/s11274-019-2731-9. PMid: 31552479.
[16] Nunes JC, Cristóvão RO, Freire MG, et al. Recent strategies and applications for L-asparaginase confinement. Molecules 2020;25(24):5827. https://doi.org/10.3390/molecules25245827. PMid: 33321857.
[17] Nguyen HA, Su Y, Lavie A. Design and characterization of *Erwinia chrysanthemi* L-asparaginase variants with diminished L-glutaminase activity. J Biol Chem 2016;291(34):17664–76. https://doi.org/10.1074/jbc.M116.728485. PMid: 27354283.
[18] Ebrahimipour GH, Movahed MG. An overview of asparaginase enzyme features: An anticancer enzyme with high potency. J Shahid Sadoughi Univ Med Sci 2020;27(12):2132–48. https://doi.org/10.18502/ssu.v27i12.2830.
[19] Pillaca-Pullo O, Rodrigues D, Sánchez-Moguel I, et al. Recombinant L-asparaginase production using *Pichia pastoris* (*MU^{T}s* strain): Establishment of conditions for growth and induction phases. J Chem Technol Biotechnol 2021;96(1):283–92. https://doi.org/10.1002/jctb.6540.
[20] Fonseca MH, Fiúza TTS, de Morais SB, et al. Circumventing the side effects of L-asparaginase. Biomed Pharmacother 2021;139:. https://doi.org/10.1016/j.biopha.2021.111616. PMid: 33932739111616.
[21] Kumar R, Sedolkar VK, Triveni AG, et al. Isolation, screening and characterization of L-asparaginase producing fungi from medicinal plants. Int J Pharm Pharm Sci 2016;8(1):281–3.

[22] Belén LH, Beltrán Lissabet JF, de Oliveira R-Y, et al. Immunogenicity assessment of fungal L-asparaginases: An in silico approach. SN Appl Sci 2020;2:222. https://doi.org/10.1007/s42452-020-2021-z.

[23] Naser S, Saber W, El-Metwally M, et al. Fungal assembly of L-asparaginase using solid-state fermentation: A review. Biocell 2020;44(2):147–55. https://doi.org/10.32604/biocell.2020.09522.

[24] Golbabaie A, Nouri H, Moghimi H, et al. L-asparaginase production and enhancement by *Sarocladium strictum*: *In vitro* evaluation of anti-cancerous properties. J Appl Microbiol 2020;129(2):356–66. https://doi.org/10.1111/jam.14623. PMid: 32119169.

[25] Gulati R, Saxena RK, Gupta R. A rapid plate assay for screening L-asparaginase producing micro-organisms. Lett Appl Microbiol 1997;24(1):23–6. https://doi.org/10.1046/j.1472-765X.1997.00331.x. PMid: 9024001.

[26] Reynolds J. Serial dilution protocols. American Society for Microbiology: Washington, DC, USA. 2005:1–7.

[27] Gonçalves AB, Maia AC, Rueda JA, et al. Fungal production of the anti-leukemic enzyme L-asparaginase: From screening to medium development. Acta Scientiarum Biol Sci 2016;38(4):387–94. https://doi.org/10.4025/actascibiolsci.v38i4.32993.

[28] Doriya K, Kumar DS. Isolation and screening of L-asparaginase free of glutaminase and urease from fungal sp. 3 Biotech 2016;6:239. https://doi.org/10.1007/s13205-016-0544-1. PMid: 28330312.

[29] Imada A, Igarasi S, Nakahama K, et al. Asparaginase and glutaminase activities of micro-organisms. Microbiology 1973;76(1):85–99. https://doi.org/10.1099/00221287-76-1-85. PMid: 4723072.

[30] Nakahama K, Imada A, Igarasi S, et al. Formation of L-asparaginase by Fusarium species. Microbiology 1973;75(2):269–73. https://doi.org/10.1099/00221287-75-2-269. PMid: 4706969.

[31] Hall BG. Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 2013;30(5):1229–35. https://doi.org/10.1093/molbev/mst012. PMid: 23486614.

[32] Tamura K, Stecher G, Kumar S. MEGA11: Molecular evolutionary genetics analysis version 11. Mol Biol Evol 2021;38(7):3022–7. https://doi.org/10.1093/molbev/msab120. PMid: 33892491.

[33] Nguyen HA, Su Y, Zhang JY, et al. A novel L-asparaginase with low L-glutaminase coactivity is highly efficacious against both T-and B-cell acute lymphoblastic leukemias *in vivo*. Cancer Res 2018;78(6):1549–60. https://doi.org/10.1158/0008-5472.CAN-17-2106. PMid: 29343523.

[34] Freitas M, Souza P, Cardoso S, et al. Filamentous fungi producing L-asparaginase with low glutaminase activity isolated from Brazilian Savanna soil. Pharmaceutics 2021;13(8):1268. https://doi.org/10.3390/pharmaceutics13081268. PMid: 34452229.

[35] Nafisaturrahmah A, Susilowati A, Pangastuti A. Screening of L-asparaginase-producing endophytic bacteria from mangrove *Rhizophora mucronata*. In: IOP Conference Series: Earth and Environmental Science. 2nd International Conference on Tropical Wetland Biodiversity and Conservation; 23-24th October 2021; Banjarbaru City, Indonesia. 2022;976(1):012042. https://doi.org/10.1088/1755-1315/976/1/012042.

[36] Kumar M, Shukla PK. Use of PCR targeting of internal transcribed spacer regions and single-stranded conformation polymorphism analysis of sequence variation in different regions of rRNA genes in fungi for rapid diagnosis of mycotic keratitis. J Clin Microbiol 2005;43(2):662–8. https://doi.org/10.1128/JCM.43.2.662-668.2005. PMid: 15695661.

[37] White TJ, Bruns T, Lee SJ, et al. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 1990;18:315–22. https://doi.org/10.1016/B978-0-12-372180-8.50042-1. PMid: 1696192.

[38] Ashok A, Kumar DS. Laboratory scale bioreactor studies on the production of L-asparaginase using *Rhizopus microsporus* IBBL-2 and *Trichosporon asahii* IBBLA1. Biocatal Agric Biotechnol 2021;34:. https://doi.org/10.1016/j.bcab.2021.102041102041.

[39] Arima K, Sakamoto T, Araki C, et al. Production of extracellular L-asparaginases by microorganisms. Agric Biol Chem 1972;36(3):356–61. https://doi.org/10.1271/bbb1961.36.356.

[40] Correa HT, Vieira WF, Pinheiro TM, et al. L-asparaginase and biosurfactants produced by extremophile yeasts from Antarctic environments. Ind Biotechnol 2020;16(2):107–16. https://doi.org/10.1089/ind.2019.0037.

[41] Sakamoto T, Araki C, Beppu T, et al. Partial purification and some properties of extracellular Asparaginase from *Candida utilis*. Agric Biol Chem 1977;41(8):1359–64. https://doi.org/10.1271/bbb1961.41.1359.

[42] Momeni V. Enhancement of L-asparaginase production by *Candida utilis* in a 13L fermenter and its purification. Int J Eng 2015;28(8):1134–9.

[43] Moguel IS, Yamakawa CK, Brumano LP, et al. Selection and optimization of medium components for the efficient production of L-asparaginase by *Leucosporidium scottii* L115—A psychrotolerant Yeast. Fermentation 2022;8(8):398. https://doi.org/10.3390/fermentation8080398.

[44] Udayan E, Kathiravan A, Gnanadoss JJ. The nutritional and cultural conditions in shake flask culture for improved production of L-Asparaginase from endophytic fungus *Fusarium* sp. LCJ324: A sequential statistical method. Ital J Mycol 2023;52(1):62–75. https://doi.org/10.6092/issn.2531-7342/16067.

[45] Rajesh MJ, Rajesh L, Veni VS, et al. Effect of inducers and physical parameters on the production of L-asparaginase using *Aspergillus terreus*. J Bioprocess Biotech 2011;1(04):110. https://doi.org/10.4172/2155-9821.1000110.

[46] Varalakshmi V, Raju KJ. Optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782 using bajra seed flour under solid state fermentation. Int J Res Eng Technol 2013;2(09):121–9. https://doi.org/10.15623/ijret.2013.0209020.

[47] Abo-Stait HM, Easa SM, Zahra FA, et al. Biosynthesis and characterization of a novel *penicillium janthinellum Biourge* L-asparaginase as a diverse biological activities agent. Egypt Pharm J 2021;20(3):180–92. https://doi.org/10.4103/epj.epj_3_21.

[48] Ali D, Ouf S, Eweis M, et al. Optimization of L-asparaginase production from some filamentous fungi with potential pharmaceutical properties. Egypt J Bot 2018;58(3):355–69.

[49] Osama S, El-Sherei MM, Al-Mahdy DA, et al. Optimization and characterization of antileukemic L-asparaginase produced by *Fusarium solani* endophyte. AMB Express 2023;13(1):96. https://doi.org/10.1186/s13568-023-01602-2. PMid: 37702815.

[50] Yap LS, Lee WL, Ting AS. Optimization of L-asparaginase production from endophytic *Fusarium proliferatum* using OFAT and RSM and its cytotoxic evaluation. J Microbiol Methods 2021;191:. https://doi.org/10.1016/j.mimet.2021.106358. PMid: 34743930106358.

[51] Zia MA, Bashir R, Ahmed I, et al. Production of L-asparaginase from *Aspergillus niger* using agro wastes by-products in submerged fermentation process. J Teknologi 2013;62(2):47–51. https://doi.org/10.11113/jt.v62.1879.