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Isolation and Screening of L-asparaginase Producing Fungi from Soil Samples of Different Rainforest Agroecological Zones of Oyo State, Nigeria

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Research Article

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ABSTRACT

L-asparaginase is an enzyme that converts L-asparagine to L-aspartate and ammonia and produced by various microorganisms which it used for the treatment of Acute Lymphoblastic Leukemia (ALL) and other malignant cancers as well as starchy food industry. Isolation and screening of L-asparaginase producing fungi from soil samples from different rainforest agroecological zones in Oyo state was done. The primary screening by qualitative methods using rapid plate assay determined that out of 83 fungal isolates, only 7 fungi showed L-asparaginase activity on plate method assay by pink colour zones. These seven isolates that preliminary screened were grown in liquid media for static and submerged fermentation and then used for secondary screening by nesslerization method. Among seven isolates, it was showed that isolate from EZ (Egbeda Zone) (EZ26A and EZ26B) showed higher values in enzyme activity, amount of ammonia and absorbance value of 58.7 UI/mL, 111.7 µmol/L 1.286 A and 59.3 UI/mL, 112.9 µmol/L 1.432 A and the highest microbial load counts were determined in AZ (akinyele zone) with (76) 7.6×10^2 for dilution factor of 10^{-1} and EZ with (24) 2.4×10^4 for dilution factor of 10^{-3}.

Keywords: Enzyme; Microorganisms; Treatment; Industry; Fungi; Ammonia; Activity

INTRODUCTION

Enzymes are proteins that act upon substrate molecules and decrease the activation energy necessary for chemical reaction to occur by stabilizing the transition state. This stabilization speeds up reaction rates and allow them to happen at physiologically significant rates. Sometimes, the enzymes build some substances and break others down. Enzymes are produced by microorganisms which are used for medical and industrial purposes.

L-asparaginase is an enzyme that converts L-asparagine to L-aspartate and ammonia and it is recommended as medical treatment of ALL and other malignant cancers [1]. The production of this enzyme is mainly preceded by submerged fermentation method. Several researchers have studied the isolation of microbial strains that produce this important enzyme, such as *Pseudomonas fluorescens*, *Serratia marcescens*, *Escherichia coli*, *Erwinia carotovora*, *Proteus vulgaris*, *Saccharomyces cerevisiae*, *Karnatakensis Streptomyces, Streptomyces venezuelae* and several species of fungi as *Aspergillus, Penicillium* and *Fusarium*. Searching from different enzyme sources specifically eukaryotic microorganisms can lead to enzymes with less adverse effects and different features compared to prokaryotic microorganisms. Some research on using bacteria and fungi isolate for enzyme production have been investigated and have reported positive results by using bacterial and fungal isolate of different genus with positive enzymatic activity.

L-asparaginase enzymes in the food industry are used as an admixture to reduce the acrylamide produced by the high temperature in starchy foods and reduce the risk of cancer [2]. This enzyme is one of the most important biochemical therapeutic enzymes used in the treatment of various types of leukemia, such as acute lymphoblastic leukemia in children [3]. However, the high specificity of L-asparaginase formulations for glutamine, low thermostability, and blood clearance are the major disadvantages [4]. L-asparaginase (L-ASNase) is a commercially essential enzyme that catalyzes the hydrolysis of L-asparagine (L-ASN) into L-aspartic acid and ammonia [5]. It is commonly found in animals, plants and microorganisms such as (fungi and bacteria) [6,7]. The enzyme obtained as asparaginase is primarily used in the treatment of ALL, hematological and non-hematological disorders [8]. It is also used in food industry to neutralize carcinogenic acrylamide and as a biosensor to detect asparagine levels during chemotherapy [9].

The long-term outcome of ALL has improved dramatically during the last few decades because of the development of effective treatments and well-designed treatment procedures. Long-term, event-free, survival rates in children are currently around 80%, and overall survival rates are close to or exceeding 90%. Although overall survival rates in adults have improved in recent years, only 38% to 50% achieve long-term survival [10,11].

L-asparaginase targets cancer cells by starving them of asparagine in which cancer cells lack asparagine synthetase and hence cannot carry out *de novo* synthesis of asparagine. Thus, L-ASNase activity causes depletion of asparagine in blood, resulting in the inhibition of protein synthesis for cancer cells [12]. L-ASNase is also a promising acrylamide mitigating agent that reduces carcinogenic acrylamide production in food industry. The carcinogenic acrylamide is produced through the maillard reaction when starch-rich foods such as (potato fries, cookies) are treated above 100°C [12].

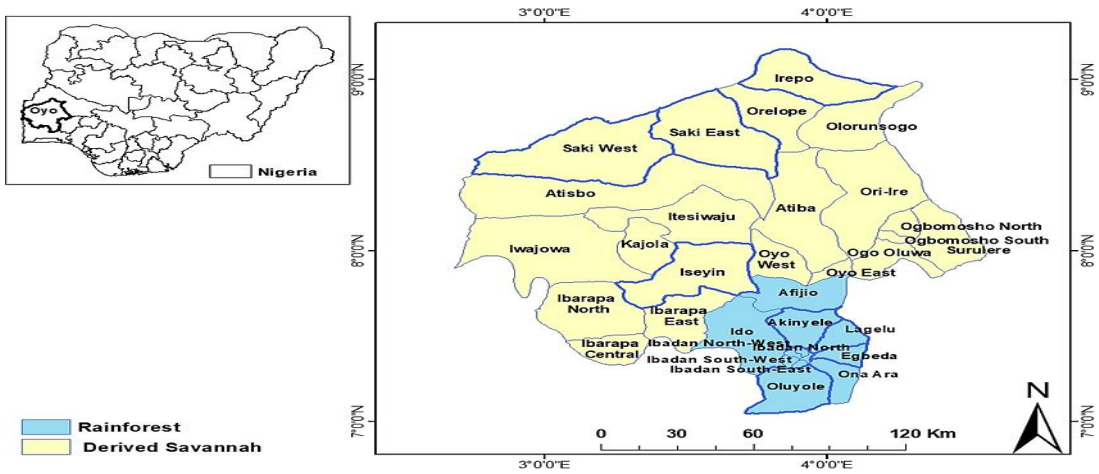
Different challenges are associated with the current applications of this versatile enzyme. One of the major challenge is in the pharmaceutical application where glutaminase activity of asparaginase results in hypersensitivity reactions in ALL patients [13]. The inevitable use of L-ASNase in pharmaceutical as well as food industry and constraints associated with the commercially available formulations require exploration of new sources of L-ASNase with an improved activity that could be produced through commercially viable processes [14]. Considering this demand of glutaminase free L-ASNase with improved biochemical and enzymatic properties for therapeutic applications. The objective of this study was to isolate the potent fungal strain producing large amount of L-asparaginase with maximum enzyme activity.

MATERIALS AND METHODS

Study area

This research work was carried out in Oyo state, southwest Nigeria. Oyo state comprises of two main agroecological zones which include, the derived savannah and the rainforest. The derived savannah comprises of local government areas such as Saki east, Saki west, Irepo, Iseyin, Oyo east, Oyo west, Ogbomosho north and Ogbomosho south. It supports the cultivation of crop such as maize, yam, cassava, cashew, soybean, pepper, mango and shea butter. The rainforest agroecology is mainly found in Ibadan area, covering local government areas including Oluyole, Akinyele, Ona-ara, Egbeda and Lagelu (Figure 1). The agroecology also favors production of food crops such as cassava, yam, sweet potato and maize and tree crops such as cocoa, citrus, and palm oil. The rainfall pattern in Oyo state is bimodal running from March to October and a dry season accompanied by the northeast trade wind with harmattan and dust from the Sahara desert is experienced between november and february [15,16].

Figure 1. Oyo state agroecological zones. **Note:** Rainforest; Derived savannah.



Sample collection

About twenty-five soil samples were collected from different places at a depth of 10 cm to 15 cm in and around the five local government areas made up of rainforest agroecology zone in Oyo state, Nigeria. The samples were collected into a sterile polythene bags and carried to department research laboratory of Oyo state college of agriculture and technology, Igboora Oyo state for further microbial analysis.

Media preparation

About 15.6 g of Potato Dextrose Agar (PDA) was weighed in a sterile conical flask and mixed with 400 mL of distilled water and shake vigorously till powdery particles dissolved. To avoid the bacteria contamination streptomycin (50 µg/mL) was added to the medium and autoclaving for 20 minutes at 121°C for fungi isolation.

Isolation of microorganisms

About 9 mL of sterile water was prepared in six test tubes for each soil sample and 1 g of each soil sample was added into the first test tube and serial dilution was taken for the rest test tubes. Pour plate method was used for

fungi isolates with the dilution factors of 10^{-1} and 10^{-3} and incubated at 28°C for 3 days–5 days. After incubation, the viable organisms were counted for fungi in Cfu/g.

Qualitative screening of L-asparaginase producing microorganisms

The isolated fungi were screened for their ability to produce asparaginase. Mycelial plug was inoculated onto modified fermentation medium in 400 mL, agar/agar powder (7.2 g/L^{-1}), glucose (0.8 g/L^{-1}), L-asparagine (4.0 g/L^{-1}), KH_2PO_4 (0.61 g/L^{-1}), KCl (0.21 g/L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.21 g/L^{-1}), $CuNO_3 \cdot 3H_2O$ (0.012 g/L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.02 g/L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.012 g/L^{-1}) and 0.00036% phenol red dye (indicator). Controls were prepared by inoculating mycelial plugs on fermentation medium without asparagine. Duplicates for each isolate were prepared. All petri plates were incubated at 28°C. After 5 days of incubation, the diameter of the pink zone was evaluated [17].

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Identification of microorganism

Identification of selected L-asparaginase producing fungal strain by their macro and micro characteristics was determined *via* fungi atlas.

Production of L-asparaginase

The fungal isolates were subcultured and transferred into fermentation medium without (agar and phenol red) in which fungi isolate was stabbed with 7 mm cork borer and shake very well in a MacCanty bottle and leave at a room temperature for 4 days. Filter paper was used to filtrate the supernatant (crude enzyme) from the mixture after 4 days incubation.

Estimation of L-asparaginase activity

The determination of L-asparaginase activity was carried out. In this assay, the rate of hydrolysis of L-asparagine was determined by measuring the released ammonia using nesslers reagent. The L-asparaginase positive fungal isolates were inoculated using 7 mm fungal mycelial plugs into 200 mL of fermentation broth and incubated for 4 days at 38°C. L-asparaginase was estimated by nesslerization as described [18]. After incubation, 500 µl of crude enzyme with 500 µl of L-asparagine solution and 500 µl of Tris Hcl buffer were mixed together and the mixture was incubated at 37°C for 30 minutes. The enzyme activity was stopped by adding 500 µl of 1.5 M Trichloroacetic Acid (TCA).

About 1000 µl of crude enzyme, 3.75 mL of distilled water and 200 µl of Nesslers reagent was added to the mixture and incubate at a room temperature for 10 minutes in which colour changes was observed. Then the

mixture was taken for analysis through spectrophotometer at 450 nm absorbance and the final solution was recorded

RESULTS

Estimation of L-asparaginase activity

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About 1000 µl of crude enzyme, 3.75 mL of distilled water and 200 µl of nesslers reagent was added to the mixture and incubate at a room temperature for 10 minutes in which color changes was observed (Tables 1-4). Then the mixture was taken for analysis through spectrophotometer at 450 nm absorbance and the final solution was recorded (Figure 2).

Table 1. Isolation of L-asparaginase producing fungi from different rainforest agroecological zones in Oyo state.

Rainforest agroecological zone	Location code	Total number of Fungi isolates
Akinyele zone	AZ	25
Egbeda zone	EZ	31
Lagelu zone	LZ	23
Oluyole zone	OZ	20
Ona-ara zone	NZ	28
Total		127

Table 2. Microbial load counts of each rainforest agroecological zone soil samples in Oyo state, Nigeria.

Rainforest agroecological zone	Location code	Fungal isolates dilution factor	
		10^{-1}	10^{-3}
Akinyele zone	AZ	(76) 7.6×10^2	(5) 5×10^3
Egbeda zone	EZ	(28) 2.8×10^2	(24) 2.4×10^4
Lagelu zone	LZ	(23) 2.3×10^2	(20) 2.0×10^4
Oluyole zone	OZ	(51) 5.1×10^2	(5) 5×10^3
Ona-ara zone	NZ	(33) 3.3×10^2	(3) 3×10^3

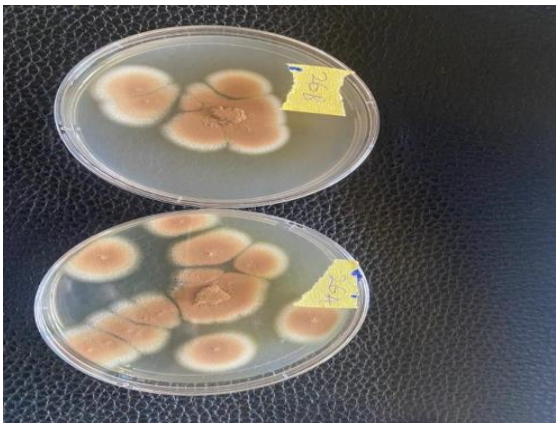
Table 3. Screening of microorganisms for L-asparaginase production.

Fungi			
Location code	**Diameter of organism**	**Diameter of organism**	**Index value**
OZ05	1.2	1.7	1.42
AZ07	1	2.7	2.70*
NZ11	1.3	3.3	2.54*
AZ17	1.1	2.9	2.64*
EZ17	2.1	4.3	2.05
LZ19	3.1	7.3	2.35*
EZ26	0.6	3	5.00*
AZ29	1.8	3.3	1.83
LZ31	1.5	3	2
NZ33	1	2	2
OZ39	1.5	4.6	3.07*
AZ41	2	4.3	2.15
EZ49	1.3	3	2.31*
OZ60	2.6	4.7	1.81
LZ61	1.9	2.9	1.53
AZ63	3.1	5.2	1.68
EZ68	0.6	1	1.67
NZ73	2.3	4	1.74
Note: ∗:-The best potent fungi isolate			

Table 4. Fungal isolates showing excellent L-asparaginase activity through spectrophotometer at (450 nm absorbance).

Rainforest agroecological zone	Location code	Absorbance value	L-asparaginase activity (UI/mL)	Ammonia amount (µmol/L)
Akinyele zone	AZ7A	0.125 A	33.5	93.4
Akinyele zone	AZ7B	0.141 A	31.4	92.7
Ona-ara zone	NZ11A	0.517 A	31.1	92
Ona-ara zone	NZ11B	0.623 A	31.3	92.1
Akinyele zone	AZ17A	0.117 A	41.7	97.7
Akinyele zone	AZ17B	0.104 A	39.9	96.3
Lagelu zone	LZ19A	0.181 A	28	78
Lagelu zone	LZ19B	0.215 A	27.9	75.9
Egbeda zone	EZ26A	1.286 A*	58.7	111.7
Egbeda zone	EZ26B	1.432 A*	59.3	112.9
Oluyole zone	OZ39A	0.061 A	35.2	97.4
Oluyole zone	OZ39B	0.020 A	35.1	97.1
Egbeda zone	EZ49A	0.110 A	29.3	78.3
Egbeda zone	EZ49B	0.010 A	28.5	78
Control	CZ	0.000 A	11.7	33.7
Note: ∗:-The best potent fungi isolate				

Figure 2. Shows the best potent fungi isolates.



DISCUSSION

A total number of 83 fungal isolates were screened for L-asparaginase production from five different agroecological zones soil samples in Oyo state using Potato Dextrose Agar (PDA) medium and incubated at 28°C for 3 days– 5 days. After incubation, the viable organisms were counted for fungi in Cfu/g. The highest microbial load counts were determined in Akinyele zone (AZ) with (76) 7.6×10^{2} for dilution factor of 10^{-1} and Egbeda zone (EZ) with (24) 2.4×10^{4} for dilution factor of 10^{-3} most of the fungal isolates have found to be positive for L-asparaginase production during screening process. Out of 83 fungal isolates, only 7 fungi showed L-asparaginase activity on plate method assay by pink colour zones. Furthermore, these isolates were subjected to the secondary screening for enzyme activity by a method [17]. Among seven isolates, it was showed from table 4 that isolate from Egbeda zone (EZ26A and EZ26B) showed higher values in enzyme activity, amount of ammonia and absorbance value of 58.7 UI/mL, 111.7 µmol/L 1.286 A and 59.3 UI/mL, 112.9 µmol/L 1.432 A respectively while other fungal isolates showed low enzyme activity, amount of ammonia and absorbance values. The enzymatic value is vital to evaluate the efficiency of the enzyme. In addition, the amount of enzyme is directly proportional to the absorbance value and the amount of ammonia. The greater the L-asparaginase enzyme activity, the greater the enzyme activity degrading L-asparagine into L-aspartate and ammonia. The greater the ammonia formed, the more concentrated the solution, resulting in a higher absorbance value.

CONCLUSION

From this research, it was clearly shows that all five agroecological zones in Oyo state soil samples can provide a rich source of L-asparaginase producing fungi but Egbeda zone produced the highest enzyme activity compared to other zones. L-asparaginase obtained from different sources is of great importance agroecological zones in Oyo state slightly differ in physical and biochemical properties with different kinetic parameters. The soil sources which provide L-asparaginase that works efficiently at 28°C to 37°C which is the normal human body temperature, hence L-asparaginase from these sources can be purified and used in the pharmaceutical industry to make drugs for human, while the highly thermostable version of this enzyme can be applied in industrial processes where high temperature is usually required. Despite the fact that L-asparaginase is being widely used as an anti-cancerous drug and in the industrial process for food treatment, it has some limitations which affect its stability and activity, therefore, there is a need of new modifications in it at the molecular level through enzyme engineering technology to obtain highly versatile versions of the enzyme.

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