NEW PRODUCTION METHOD OF BACTERIAL L-ASPRAGINASE ENZYME FROM DIFFERENT SOIL ENVIRONMENTS IN EGYPT AS ANTICANCER AGENT

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ABSTRACT:

Background: *Acute lymphoblastic leukemia (ALL) is a hematologic cancer that preponderantly falls out in children betwixt 2 and 10 years of age. L-asparaginase is a constitutional element of management for long-suffering with ALL. L-asparagine degrading enzymes are necessary for the treatment of auxotrophic cancers such as acute lymphoblastic leukaemia. Auxotrophic cancers have not the ability to synthesize asparagine but normal cells can synthesize it. Asparagine degrading enzymes include asparaginase enzyme.*

The aim of the study: *our study was concerned with isolation and screening of bacterial producing L-asparaginase enzyme as anticancer agent for treatment of auxotrophic cancers from different soil environments. Also, concerned with determination of environmental and physiological factors affecting the growth of some L-asparaginase producing bacterial isolates. Characterization of L-asparaginase enzyme was also included in our study.*

The type of the study: *Screening experimental study.*

Methodology: *In our study some bacterial isolates were analyzed for production of L-asparaginase on mineral asparagine agar selective media (MAA). These bacterial isolates which showed positive growth on MAA utilized the asparagine as the sole metabolic source of carbon and nitrogen for their growth. Direct Nesslerization test was used for detection and screening of the presence of bacterial asparagine degrading enzymes via their ability to produce ammonia from degrading the asparagine in different dilutions of soil samples.*

Results and discussion: *The optimal environmental and physiological factors affecting growth of these positive isolates were PH 7.3 at temperature 37c in aerobic conditions. The morphological and the biochemical tests revealed that Bacillus subtilis and Bacillus anthracis were the major positive bacterial producing L-asparaginase isolates of soil samples collected from different soil environments.*

Conclusion: *Characterization of bacterial L-asparaginase production as anticancer agent in Egypt included activators of the enzyme production :0.5 g/l KCL,0.5 g/l MGSO4,1.0 g/l FeSO4,0.1 g/l ZnSO4 at PH 7.3 at temperature 37 under aerobic conditions.*

Keywords: *Microbial L-asparaginase, production, anticancer agent.*

INTRODUCTION:

Acute lymphoblastic leukemia (ALL) is a worldwide challenge. It is a hematologic malignity that preponderantly passes off in children between 2 and 10 years of age. L-asparaginase is an integral constituent of treatment for patients with ALL.[1] ALL, a combative form of blood malignant

neoplasm predicted to trouble over 53,000 individual globally.[2]

Difference between benign and malignant tumors:

Malignant tumors are neoplasm(cancer), while benign tumors are not cancer.[3]

A. Benign tumors do not spread to other tissues and Locally being grown. They may represent danger if they badly affect vital organs like the brain.[4]

B. Malignant tumors can invade and spread to other tissues. This is called metastasis.[5]

Cancer metastasis:

A. It is the process which involves the spreading and invading of neoplastic cells other tissues of the body.[6]

B. Neoplastic cells travel to other sites by the blood and the lymphatic system.[7]

C. It is crucial as it aids in determining the staging and the treatment.

D. Metastatic neoplasms are secondary neoplasms as their origin is from primary cancer.[8]

Causes of cancer:

A. Genetic mutations:

Neoplastic cells are caused DNA damage and out of control cell growth.

Examples: Mutations of genes BCRA1 and BCRA2.Both contribute to increased risk of breast and ovarian cancers.[9]

B. Environmental causes:

Sun light exposure can cause cancer through ultraviolet radiation, also, air pollutants can cause cancer.[10]

C. Microbe cause:

Several microbes may increase the risk of occurrence of neoplasms.

Examples:

Helicobacter pylori is accompanied with gastric cancer.

Human papulomavirus (HPV) is associated with cervical cancer.

Hepatitis B and C viruses increase the risk of hepatic carcinoma.[11]

D. Life style and diet causes:

1- Eating a poor diet, inactivity increase the risk of cancer occurrence.

2- Obesity, heavy smoking, heavy alcohol use and exposure to chemicals and toxins increase the risk of neoplasm.[12]

E. Radiation and chemotherapy treatment:

Both can cause damage to the healthy cells and consequently increase the risk of cancer occurrence.[13]

Auxotrophic cancer cells deficient in L-asparagine biosynthesis:

Auxotrophic cancer cells such as acute lymphoblastic leukaemic cells can not synthesize L-asparagine amino acid which is essential for their survival and growth but normal cells can synthesize it. Deprivation of these cancer cells from L- asparagine by bacterial L-asparaginase is of great value.[14]

Physiologic importance of L-asparagine for cell biology in cancer cells:

L-Asparagine is necessary for acute lymphoblastic cancers as a sole metabolic source of carbon and nitrogen .

Degradation of L-asparagine amino acid:

It is degraded by L-asparaginase yielding L-aspartic acid and ammonia.[15]

AIM OF THE STUDY:

Auxotrophic cancer cells such as acute lymphoblastic leukaemia are leading causes of death to human,so our study is to search new treatments for them such as bacterial

L-aspraginase enzyme from different soil environments in Egypt.

Protocol of study:

1- Collecting grassland soil samples from different environments.
2- Isolation of L-asparaginase producing bacteria on selective medium(MAA).
3- Determination of physiologic and environmental factors affecting the growth of some selected bacterial isolates producing L-aspaarginase.
4- Characterization of L-asparaginase production and activity by some selected bacterial isolates.

MATERIAL AND METHODS:

Type of the study:

Screening experimental study.

Date and place of the study: Faculty of pharmacy, Cairo university, Egypt during May 2021.

1- Material:

a. list of media:

1. Identification and isolation media:

a-Mineral Asparagine Agar (MAA):

A selective media for the isolation and identification of microorganisms having the ability to use L-asparaginase as the sole carbon and nitrogen metabolic source. This medium composed of the following ingredients(g/L). All ingredients were bought from Algomhuria company for chemicals in Cairo, Egypt

Table 1. Ingredients of Mineral Argenine Agar.

Ingredient	Unit of measurement
Potassium chloride	0.5 mg
Magensium sulfate	0.5 mg
KH2po4	1.0 g
Ferrous sulfate	0.1 g
Zinc sulfate	0.1 g
L.asparagine	1.0 g
agar	2%

The ingredients mixed together, dissolved in boiling water, agitated frequently until completely dissolved. Then the medium cooled in water bath to 47-50 c and PH of the medium was at 7.3 at temperature 37c.Directly after mixing the ingredients the plates were poured up to volume of 15-20 ml in dishes with a diameter of 90 mm.

b-Blood agar:

An enrichment media for identification of beta- hemolysis of the positive isolates cultured on MAA to aid in identification of L. asparaginase producing bacterial species together with the gram stain, the biochemical tests and the morphology of colonies.

Composition of blood agar

Table 2. list of ingredients of blood agar.

ingredient	Unit of measurement
peptone	10.0 g/L
tryptose	10.0 g/L
Sodium chloride	5.0 g/L
Agar	15.0g/L
Distilled water	960 ml

Final PH at 25 c :7.3

The ingredients were combined then 5% sheep blood was added after autoclaving at 121 c for 15 minutes and before pouring onto the plates.

All ingredients were bought from Algomhuria company for chemicals in Cairo, Egypt

b. **Uv detection of production of Egyptian soil bacterial cultures containing L-asparagine degrading enzymes**:

Using direct nesslerization test for the estimation of optical density of metabolic nitrogenous containing compound (Ammonia) due to degrading asparagine present in media by bacterialL- asparagine degrading enzymes under PH7, Temp 37 c, .05 borate buffer and traces of Ni, Co and Mn metal ions as cofactors. All ingredients

were bought from Algomhuria company for chemicals in Cairo,Egypt

Ethical statement:

In the present study, we followed All applicable national, international and/or institutional guidelines for the attention and utilization of humans and animals. All processes carried out in study including humans and animals were authorized by the local authorities, Ethical committee for human and animal handling at Cairo university(ECAHCU), at the faculty of Pharmacy, Cairo University, Egypt in agreement with the recommendations of the weathrall report with approval number P-13-1-2022. All efforts were performed to ablate the number of humans and animals utilized and their suffering during study.

2-Equipment

Table 3. List of instruments

Instrument	Model and manufacturer
Autoclaves	Tomy,japan
Aerobic incubator	Sanyo,Japan
Digital balance	Mettler Toledo,Switzerland
Oven	Binder,Germany
Deep freezer -80	Artiko
Refrigator 5	whirpool
PH meter electrode	Mettler-toledo,UK
Deep freezer -20	whirlpool
Gyratory shaker	Corning gyratory shaker,Japan
190-1100nm Ultraviolet visible spectrophotometer	UV1600PC,China
Light(optical) microscope	Amscope 120X-1200X,China

3-Methods:

a. Sample collection:

50 Samples were grassland soil collected from the 0-10 cm depth, samples collected from different areas and chosen randomly. Samples placed in sterile containers and stored at 4 c untill processed.

b. Isolation of L-asparaginase producing strains:

1. One gram of each soil sample was suspended in 99 ml of sterile distilled water contained in 250 ml erlenmeyer flasks and shaken at 400 RPM for two minutes in a gyratory shaker.
2. The soil suspensions were serially diluted in sterile distilled water and the dilutions from 1/10 to1/1000000 were plated on mineral asparagine agar(MAA) medium containing 0.5 g Kcl,0.5 g Mgso4,1 g KH2po4,0.1 g Feso4,0.1 g Znso4,1 g L.argenine and 2% agar. The PH of medium adjusted at 7.3 at temperature 37
3. The plates were incubated at 37c for 48 hours.
4. Only the microorganisms having the ability to use L-asparaginase as the sole metabolic carbon and nitrogen source can grow on the MAA medium.
5. Colonies showing growth should be purified twice using streak plate technique ,then taken on nutrient agar slants and kept at 4 c.

c. **Identification of L-asparaginase producing strains:**

1. **Gram stain:**

It separates bacteria into two classifications according to the composition of their cell walls. If a specimen on a

microscope slide is treated with a solution of crystal violet and then iodine, the bacterial cells will stain purple. If the stained cells are then treated with a solvent such as alcohol or acetone ,gram positive organisms retain the stain, where as gram negative species lose the stain, becoming colorless. Addition of counterstain safranin stains the clear, gram negative bacteria pink.

2. **Motility test under the microscope:**

It separates the bacteria into either motile or non motile.

3. Biochemical tests:

a-lecithenase test:

A loopful of the test organism is taken and is streaked on the plate. incubate at 35-37 c for 24 hours, then examine the plate for opalescent halo surrounding the inoculums(precipitation around the streak of bacteria (+ve))

b-Methyl red test:

c-Catalse test:

d-Voges-Proskauer (VP) test:

4. Subculture of the positive isolated colonies on sheep blood agar to show if there is beta-hemolysis or not

d. The determination of optimal environmental and physiological factors affecting L-asparaginase production by some selected isolates. (PH,Temprature)

e. charecterization of L-asparaginase produced by certain selected isolates

The determination of the optimal activators for the production of the L-asparaginase enzyme from the positive cultured bacterial isolates on mineral asparagine agar.

f. Uv detection of production of Egyptian soil bacterial cultures containing L-asparagine degrading enzymes:

Using direct nesslerization test for the estimation of optical density of metabolic nitrogenous containing compound(Ammonia) due to degrading asparagine present in media by bacterial L-asparaginase degrading enzymes under PH7,Temp 37 c, .05 borate buffer and traces of Ni,Co and Mn metal ions as cofactors.

RESULTS AND DISCUSSION:

RESULTS

A-Distribution of L. asparaginase producing bacteria in grassland soil samples collected from different areas

The study started by screening 50 soil samples for L-asparaginase producing bacteria. The grass land soil samples were from different areas in Egypt:

1. Menyat Sheben Elkanater, Qalyobia (n=1)
2. Eltal Bani Tamim,Sheben Elkanater , Qalyobia (n=2)
3. Monshaat Elkeram,Sheben Elkanater,Qalyobia (n=3)
4. Nawa,Sheben Elkanater,Qalyobia (n=4)
5. Kafr Sheben Elkanater,Qalyobia (n=5)
6. Elsahafa,Mashtol Elsok,Sharqia (n=6)
7. El manyer,Mashtol Elsok,Sharqia (n=7)
8. El manasra,Mashtol Elsok, Sharqia (n=8)
9. Brash,Mashtol Elsok, Sharqia (n=9)
10. Kafr Brash,Mashtol Elsok, Sharqia (n=10)
11. Eldir,Tokh, Qlayobia (n=11)
12. Arab Elhaswa, Tokh, Qlayobia (n=12)
13. Dandana,Tokh,Qlayobia (n=13)
14. Elmanzala,Tokh,Qlayobia (n=14)
15. Moshtohr,Tokh,Qlayobia (n=15)
16. Senhera,Qaha,Qalyobia (n=16)
17. Salamant,Belbis,Sharqia (n=17)
18. Menyat Salamant,Belbis,Sharqia (n=18)
19. Tal Rozon, Belbis, Sharqia (n=19)
20. Elsalam, Belbis ,Sharqia (n=20)
21. Elzawamel,Belbis,Sharqia(n=21)
22. Awlad Seif,Belbis,Sharqia(n=22)
23. Burdin,Elzagazig,Sharqia(n=23)
24. Alaslogy,Elzagazig,Sharqia(n=24)
25. Elzahraa,Elzagazig,Sharqia(n=25)

26. Alnakarya,Elzagazig,Sharqia(n=26)
27. Alreyad,Elzagazig,Sharqia(n=27)
28. Alzanklon,Elzagazig,Sharqia(n=28)
29. Alsaada,Elzagazig,Sharqia(n=29)
30. Alelwya,Elzagazig,Sharqia(n=30)
31. Aboyasin,Abokabir,Sharqia(n=31)
32. Alahraz,Abokabir,Sharqia(n=32)
33. Alrahmanya,Abokabir,Sharqia(n=33)
34. Alkramos,Abokabir,Sharqia(n=34)
35. Manshyet Radwan,Abokabir,Sharqia(n=35)
36. Algamalya,Alhusynia,Sharqia(n=36)
37. Alhegazya,Alhusynia,Sharqia(n=37)
38. Alnasrya,Alhusynia,Sharqia(n=38)
39. Bahr Albakr, Alhusynia,Sharqia (n=39)
40. Sanhagr,Sharqia (n=40)
41. Nay,Qalyob,Qalyobia (n=41)
42. Kafr Ramada,Qalyob,Qalyobia (n=42)
43. Tanan,Qalyob,Qalyobia (n=43)
44. Meet Halfa,Qalyob,Qalyobia (n=44)
45. Alsad,Qalyob,Qalyobia (n=45)
46. Balaqs,Qalyob,Qalyobia (n=46)
47. Halaba, Qalyob,Qalyobia (n=47)
48. Meet Nama,Shubra Alkhima,qalyobia (n=48)
49. Kafr Hamza, Khanka, Qalyobia (n=49)
50. Arab Alelykat,Khanka,Qalyobia (n=50)

Table 3. Prevalance of L-asparaginase producing bacteria among grassland samples

Grassland soil sample(n)	Positive isolate	Negative isolate
n1	+	
n2	+	
n3	+	
n4		-
n5	+	
n6	+	
n7	+	
n8		-
n9	+	
n10		-
n11	+	
n12		-
n13		-
n14	+	
n15	+	
n16	+	
n17	+	
n18	+	
n19	+	
n20		-
n21	+	
n22	+	
n23	+	
n24		-
n25	+	
n26	+	
n27		-
n28	+	
n29	+	
n30		-
n31	+	
n32		-
n33	+	
n34		-
n35	+	
n36		-
n37	+	
n38	+	
n39	+	
n40	+	
n41	+	
n42	+	
n43	+	
n44		-
n45	+	
n46	+	
n47	+	
n48	+	
n49	+	
n50	+	
Total	37	13

2-Morphalology, the biochemical tests and subculture of the isolates on sheep blood agar

The positive bacterial isolates (1, 3, 7, 9, 11, 14, 16, 17, 19, 22, 23, 28, 31, 33, 35, 38, 39, 42, 46, ,47, 49) on the mineral asparagine agar were observed under a light microscope gram staining. They exhibited a charecterstic morphology of gram positive, large rods, flagellated, motile. The colonies were large, irregular, yellowish white in colour and raised wrinkly colonies. They exhibited beta-hemolysis. They also, exhibited **characteristic biochemical tests:**

Table 4.

Biochemical test	Result
Catalase test	positive
Lecithinase test	negative
Methyl red	negative
Indole test	negative
Voges proskauer	positive

The positive bacterial isolates (2, 5 ,6,1 5, 18, 21, 25, 26, 29, 37, 40, 41, 43, 45, 48, 50) on the mineral asparagine agar were observed under a light microscope gram staining. They exhibited a charecterstic morphology of gram positive ,large rods ,nonmotile. The colonies were large ,irregular, greyish white in colour .They exhibited no hemolysis on blood agar. They also exhibited **charecterstic biochemical tests**:

Table5.

Biochemical test	Result
Catalase test	positive
Lecithinase test	positive
Methyl red	negative
Indole test	positive
Voges proskauer	positive

3-determination of enviromental and physiologic factors affecting growth of L.asparaginase producing bacteria.

The optimal conditions for the growth of L.asparaginase producing bacteria are PH 7.3 at temperature 37c.

4- Characterization of bacterial L. asparaginase production

The optimal conditions for the production of bacterial L.asparaginase enzyme are

a. Activators of the enzyme production:

0.5 g/L KCL,0.5 g/L MgSO4,1.0 g/L KH2PO4,0.1 g/L FeSO4,0.1 g/L ZnSO4.

b. The optimal PH and temperature for bacterial enzyme production are PH 7.3 at temperature 37c.

5-Uv detection of production of Egyptian soil bacterial cultures containing asparagine degrading enzymes:

Using direct nesslerization test for the estimation of optical density of metabolic nitrogenous containing compound (Ammonia) due to degrading asparagine present in media by bacterial asparagine degrading enzymes under PH7, Temp 37 c, .05 borate buffer and traces of Ni, Co and Mn metal ions as cofactors.

Conc. Of soil bacterial culture containing asparaginase enzyme(serial dilutions from 10*-1 to 10*-6)	Optical density (Nm) of metabolic nitrogenous compound at 425 nm by UV spectrophotmeter
0.00	0.00
1/10	0.438
1/100	0.376
1/1000	0.307
1/10000	0.226
1/100000	0.151
1/1000000	0.078

The optimal conditions for bacterial asparagine degrading enzymes production at different concentrations from 10*-1 to 10*-6 of the Egyptian soil containing bacetria producing asparaginase degrading enzymes using UV spectrophotometer are PH7.3 at

temperature 37 c and traces of activators CO, Ni, Mn.

Statistical analysis:

All cultures were conducted in triplets. Their presentation was by means and standard deviation. One way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software.

DISCUSSION:

Isolation and the screening of L-asparaginase producing bacteria:

A total of 37 bacterial isolates were isolated from different soil samples and used for various screening studies.

The current study involved the screening of isolated bacteria on mineral asparagine agar media with the asparagine as the sole metabolic source of nitrogen and carbon. Only the organisms that were able to utilize nitrogen and carbon could grow.

Identification and screening of L-asparaginase degrading enzymes:

The collected soil samples were further analyzed by nesslerization test. This method has been applied for the determination of the ammonia concentration as a product of enzymatic degradation by asparagine degrading enzymes. The concentration of the liberated ammonia by test samples demonstrated that asparagine degrading enzymes were present in collected samples. Among the collected samples, samples from 1 to 37 which were further identified as Bacillus species produced the highest amount of L-asparagine degrading enzymes.

Identification and the characterization of the most potent producing bacteria:

The morphological characteristics of isolates from 1 to 37 which showed high L-asparaginase activity indicated that the organism belonged to the genus Bacillus. The biochemical characterization by biochemical tests revealed that it has about 74% similarity with Bacillus subtilis and about 26% similarity with Bacillus anthracis. Comparing our results with another study conducted in India (Isolation, screening and characterization of L-asparaginase producing soil bacteria, July 2019), it was found that the prevalent L-asparaginase producing bacteria were Bacillus subtilis and Bacillus anthracis in our results, while it was another species in Indian study.

Figure 1.Bacterial postive isolates on MAA.

Figure 2.Positive isolates of subculture of bacterial Bacillus sp(taken from positive bacterial bacillus sp isolates on MAA) on nutrient agar plate.

Figure 3.Gram positive Bacilli.

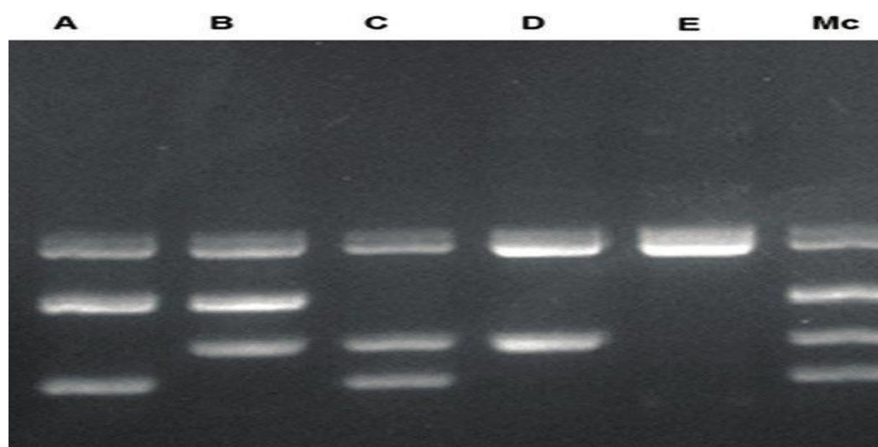


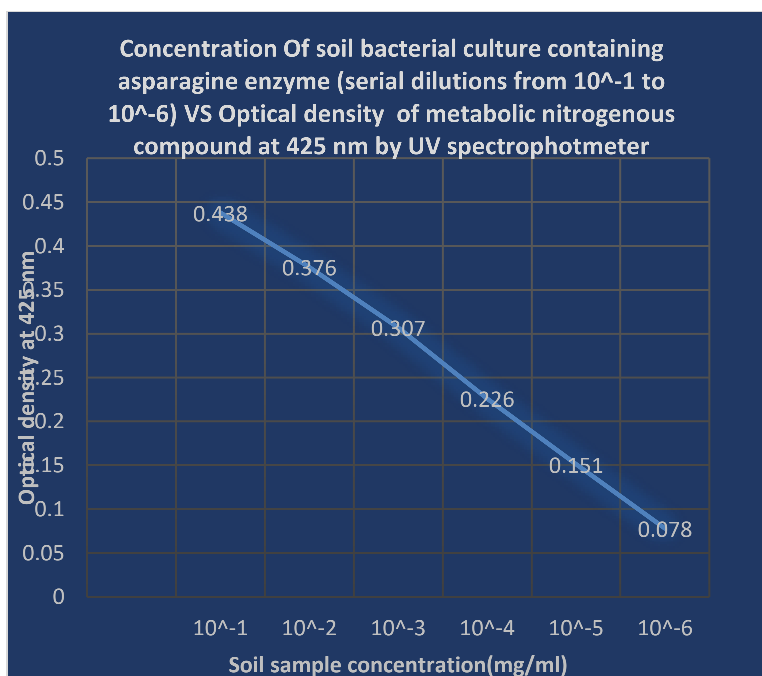
Figure 4. shows different amounts and sizes of recombinant proteins of L-aspararginase deliberated via the Western blot technique. The maximum yield of L-arginase was 75-80 mg/l.The purity of recombinant L-aspararginase was close to 86%.

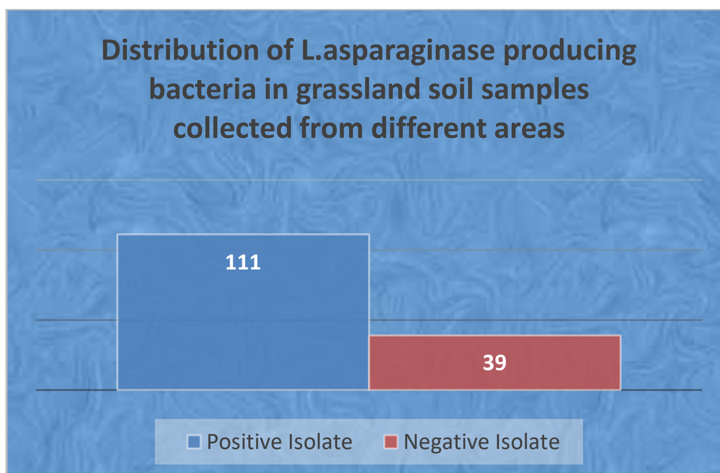
Environmental and physiological factors affecting growth of L-asparaginase producing bacteria:

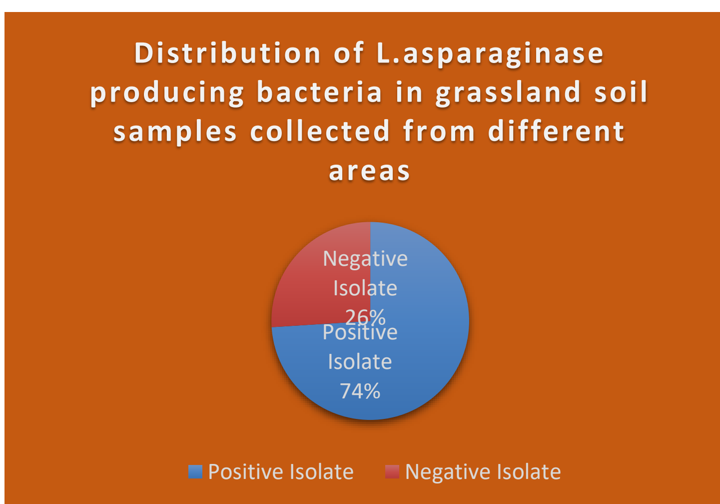
The optimal conditions for the growth were PH7.3 at temperature 37c.

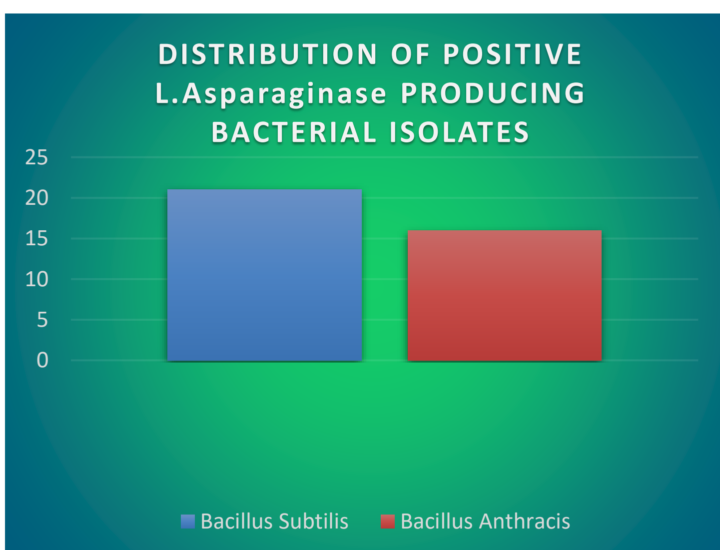
Characterization of bacterial L-asparaginase production:

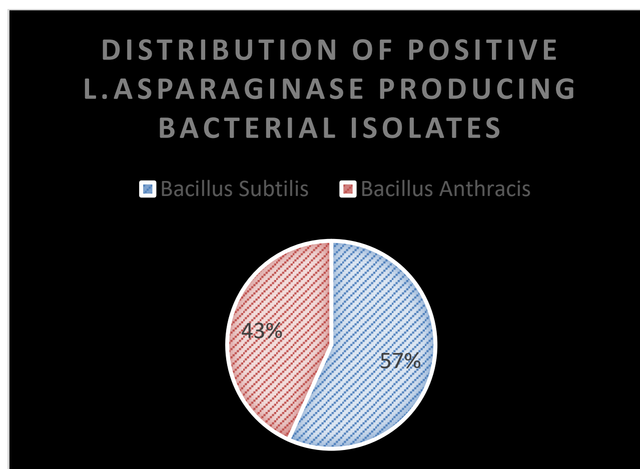
The activators of the enzyme production :0.5 g/l KCL,0.5 g/l MGSO4,1.0 g/l FESO4,0.1 g/l ZnSO4.




Distribution of L.asparaginase producing bacteria in grassland soil samples collected from different areas
111
39
Positive Isolate
Negative Isolate


Distribution of L.asparaginase producing bacteria in grassland soil samples collected from different areas
Negative Isolate 26%
Positive Isolate 74%
Positive Isolate
Negative Isolate


DISTRIBUTION OF POSITIVE L.Asparaginase PRODUCING BACTERIAL ISOLATES
25
20
15
10
5
0
Bacillus Subtilis
Bacillus Anthracis



Conclusion:

Gram positive Bacillus subtilis and anthracis showed the high amounts of bacterial asparagine degrading enzymes. This was confirmed by biochemical tests and positive isolation on mineral asparagine agar media. The optimal conditions for characterization of L-asparaginase production were with the following activators KCL, ZnSO4, FeSO4, K2HPO4, MgSO4 at PH7.3 at temperature 37c. It is recommended that in the following studies about similar topics to involve tissue culture and animal model tests to determine the optimal dose of L-aspaarginase enzyme for the treatment of auxotrophic cancers. It is also, recommended in the following studies about similar topics concerning with L-asparaginase enzyme that screening and designing the optimal dosage form for L-asparaginase enzyme as anticancer agent for auxotrophic cancers.

Conflict of interest:

There is no conflict of interest.

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طريقة انتاج جديدة لانزيم الاسبراجنيز البكتيري من بيئات تراب مختلفة في مصر

محمد كساب

محاضر علوم مايكروبيولوجي ومناعة. كلية الصيدلة جامعة القاهرة مصر.

سرطان الدم الليمفاوي الذي يصيب الاطفال مشكلة تؤرق العالم كله وانزيم الال اسبراجنيز فعال في علاجه لدى هؤلاء الاطفال المصابين به عالميا.

الانزيمات المسئولة عن تكسير الحمض الاميني الاسباراجين ضرورية لعلاج السرطانات المعتمدة على حصولها على الاسباراجين من الوسط الخارجي لانها ليس لها القدرة على تصنيعها مثل سرطانات الدم الليمفاوية بينما الخلايا العادية لها القدرة على تصنيعه.هذه الانزيمات تشمل الاسباراجنيز.دراستنا كانت معنية بعزل واستكشاف البكتريا المنتجة للاسباراجنيز كعامل مضاد للسرطان من بيئات تربة مختلفة وايضا تعيين وتقرير العوامل ابيئية والفسيولوجيةالمؤثرة على نمو بعض العزلات المنتجه للاسباراجنيز وتحديد خصائص الانزيم .تم استعمال وسط خاص لزراعة البكتريا المنتجة لانزيم الاسباراجنيز يسمى الوسط الاسباراجيني المعدني.البكتريا التي كان لها القدرة على انتاج انزيم الاسباراجنيز واستعمال الاسباراجيم كمصدر للكربون والنيتروجين لنموها هي التي نمت وعن طريق دراسة الشكل الظاهري لهذه العزلات واجراء الاختبارات الكيميائية الحيوية تبين انها بكتريا موجبة الجرام عصوية واغلبها من نوعي البكتريا سابتيليس وانثراكاس.

العوامل البيئية والفسيولوجية المؤثرة على نموالبكترياهي انها كانت من تربة الوسط بها حمضي وبجانب مطاحن الدقيق والبقوليات الغنية بالاسباراجين ودرجة حموضة الوسط الاسباراجيني المعدني كانت 7.3 عند 37 درجة مئوية في وجود الاوكسجين وتبين ان الاملاح المحفزة لانتاجه هي كبريتات الزنك والماغنسيوم والحديد وكلوريد البوتاسيوم واملاح بوتاسيوم الفوسفات الهيدروجينية.تم عمل اختبار نسلر المباشر للكشف عن البكتريا المنتجة للانزيم ونشاط الانزيم لتركيزات مختلفة من عينات التربة وتبين ان املاح المعادن المنشطة لفاعليته هي املاح كبريتات المنجنيز وثنائي كلورايد الكوبلت والنيكل عند درجة حموضة وسط معتدلة 7.3 و37 درجة مئوية في وجود الاوكسجين.