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Isolation and survey of L-asparaginase producing bacteria from soil

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ABSTRACT

L-asparaginase produced by various microorganisms which it used for the treatment of leukemia and starchy food industry. Screening of L-asparaginase producing bacteria from soil samples from Damietta different locations was done. The primary screening by qualitative methods using rapid plate assay determined that out of 25 isolates, 20 strains were producing L-asparaginase. The greatest pink color zones were observed for four bacterial isolates(A6, A11, C11 and C19) after 48 hrs. These four isolates that preliminary screened were grown in liquid media for static and submerged fermentation and then used for secondary screening by Nesslerization method. The best four isolates producing L-asparaginase were identified based on morphological, cultural and biochemical tests, as A6, C11 and C19 were identified as *Bacillus* species and A11 was identified as *Staphylococcus* species.

Key words : L-asparaginase, *Bacillus*, *Staphylococcus*.

INTRODUCTION

L-asparaginase was discovered by Clementi in 1922in serum of guinea pig (Badoei-Dalfard, 2016).L-asparaginase (EC3.5.1.1) belongs to a group of homologous amidohydrolases family, that catalyst the hydrolysis of amino acid L-asparagine to L-aspartate and ammonia (Fernandes and Gregoriadis1997). L-asparaginase aminohydrolase has gained attention in recent years due to its important applications, as its use in pharmaceutical industry as an alternative for treatment of different cancers suchas acute lymphoblastic leukemia, malignant diseases ofthe lymphoid system and Hodgkin's lymphomas (Pieters *et al*., 2011). Also, this enzyme is used in food industry to prevent theacrylamide formation when foods are processed in high temperatures. This use is important because acrylamideis a neurotoxin classified as potentially carcinogenic to humans.

L-asparaginase is present in many animal tissues, plants, microorganisms and in the serum of certain rodents but not in mankind. Although L-asparaginases are broadly distributed among various living organisms, however, microorganisms like bacteria, fungi, yeast, actinomycetes and algae are very efficient producers and the better source of L-asparaginase, because they can be cultured easily and the extraction and the purification of L-asparaginase from them are also convenient, facilitating the large scale production (Patro*et al*.,2011). L-asparaginase from *Escherichia coli* produces two types of enzyme, L-asparaginase I (EC1),found in the cytoplasm and L-asparaginase II (EC2), with periplasmic origin (Cachumba *et al*., 2016). However, only the second one has anti-cancer activity, some studies described EC1 as a constitutive enzyme and EC2 as secreted only as a response to exposure tolow concentrations of nitrogen(Charbonneau *et al*., 2017).

Most of the microbial L-asparaginase is intracellular in the nature except few which are secreted outside the cell (Narayana,2008; Rajan *et al*., 2018). Extracellular L-asparaginase is more advantageous than intracellular type because of higher accumulation of enzyme in culture broth under normal conditions, easy extraction, and downstream processing (Amena*et al.,* 2010).

Screening for bacteria producing L-asparaginase has been prospected by many methods. The activity of L-asparaginase is accompanied by increase in pH due to liberation of ammonia, presence of ammonia is used qualitatively and quantitatively to determine the enzyme activity (Mashburn and Wriston, 1963;Abdelrazek *et al.*, 2019).So this study aims to isolation and screening of L-asparaginase producing bacteria from different Damietta soil samples.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from different locations in Damietta Governorate. These soil samples were taken from places has an area of 1 m^2 in clean plastic bags. All samples were immediately transported to microbiology laboratory, Botany and Microbiology Department, Faculty of Science, Damietta University for bacterial isolation. The soil samples were processed within 1-2 hours of collection (Steubing, 1993).

Isolation and primary screening of L-asparaginase producing bacteria (qualitative assay)

The bacteria were isolated by plating dilutions in sterile distilled water throughout weighting 10 g of each soil samples and putting in a 250 mL Erlenmeyer flask containing 90 mL of sterile distilled water and shaking for 15 minutes and these suspensions were considered as 10^{-1} dilutions (Bhat *et al.*, 2015). Each sample were diluted in the range of 10^{-2} to 10^{-5} and 0.1 ml of diluents was spread on modified sterile Czapek's dox Agar medium (g/l)(glucose2.0, L-asparagine10.0, KH_2PO_4 1.52, Na_2HPO_4. $2H_2O$6.0, $MgSO_4$.$7H_2O$ 0.5, $CaCl_2.2H_2O$ 0.5, NaCl 0.5, traces from $Cu(NO_3)_2.3H_2O$, $ZnSO_4.7H_2O$ and $FeSO_4.7H_2O$,agar 15.0 and pH 6.9±0.2) and incubated at 37°C for 24 hours in the static incubator. After incubation period, the developed single colonies of bacteria which varied in shape and color were picked up and purified by streaking on fresh nutrient agar plates. The purified bacterial isolates were regularly subcultured and stored on nutrient agar slants at 4°C (Bahig *et al*., 2008). Primary screening of L-asparaginase producing bacteria was performed by rapid plate assay (Gulati *et al*., 1997). Modified M9 medium(g/l) (peptone 3.0, lactose 3.0, L-asparagine 6.0, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2.2H_2O$ 1.0, agar 15 and pH 6.5±0.2) containing 0.005% phenol red indicator was inoculated by each bacterial colony then incubated at 37°C for 24-48 hrs.and plate wasn't inoculated work as control.

Secondary screening of L-asparaginase producing bacteria (quantitative assay)

Isolated microorganisms from the previous screening were cultured in different liquid media such as TGY-Broth with 1% asparagine at pH7.0,Modified M9 broth medium at pH 6.5, Modified Czapek's dox broth medium at pH 6.9 and Asparagine dextrose salts broth medium at pH 6.8in 250 ml Erlenmeyer flasks. Uninoculated medium served as negative control. All experiments for quantitative screening were done in triplicate after incubation on a rotary shaker (37°C, 75 rpm) for 48 hrs. and static incubator 37°C for 48 hrs., the culture broth was centrifuged at 7,000 rpm for 10 min, supernatants were collected and enzyme assay was done by nesslerization (Imada *et al*., 1973; Benchamin *et al*., 2019).

L-asparaginase assay

The L-asparaginase activity was determined by hydrolysis of L-asparagine to release the ammonia which was measured by using Nessler's reaction (Yim & Kim 2019). A mixture of 0.1 ml enzyme extract, 0.2 ml of 0.05M Tris-HCl buffer (pH 8.6) and 1.7 ml of 0.01M L-asparagine

was incubated for 10 min at 37˚C. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA), and then centrifugation at 1000 rpm, 0.5 ml of supernatant was diluted to 7ml with distilled water and treated with 1 ml of Nessler's reagent. The color reaction was allowed to develop for 10 min and absorbance read at 480 nm with a spectrophotometer (Kishor *et al*., 2015). One international unit (IU) of L-asparaginase was defined as amount of enzyme that liberates one µ mole of ammonia per minute (Prakasham *et al*., 2007).

Protein Estimation

Total protein contents were estimated according to method described by Bradford (1976).

Identification of the selected bacterial strains

The best L-asparaginase producing bacteria were identified listed in according to Bergey's manual of determinative bacteriology (Sneath *et al*., 1986).

Statistical analysis

Data were statistically analyzed for variance and least significant difference using one-way analysis of variance (ANOVA). A software system SPSS version 22 was used for these calculations.

RESULTS

Isolation and primary screening of L-asparaginase producing bacteria

25 bacterial isolates were isolated from six soil samples collected from different locations of Damietta Governorate (Table 1). Based on rapid plate assay, it was observed that among 25 isolates, 20 bacterial strains showed pink color on modified M9 medium for L-asparaginase production. Four isolates from L-asparaginase producing bacteria showed better pink color zone more than any other isolates (Table 2 and Fig. 1), so these four isolates were used for fermentation study to determine the crude enzyme activity.

Secondary screeningof L-asparaginase producing bacteria

Static fermentation was performed by inoculating the four isolates bacteria(A6, A11, C11 and C19) into modified M9 medium at specific time interval of 12, 24, 36, 48, 60 and 72 hrs. and the results of enzyme activity and specific activity (Figs. 3 & 4).

Enzyme activity and specific activity were increased after 48 hrs. for all four bacterial isolates and the isolate A6 showed maximum enzyme activity (0.454U/ml) and specific activity (6.98U/mg) within 48hrs.

Submerged fermentation was performed by inoculating isolates bacteria(A6, A11, C11 and C19) bacterial isolates into different four media as follows: TGY-Broth with 1% asparagine, modified M9 broth medium, modified Czapek's dox broth medium and asparagine dextrose salts broth medium at specific time interval of 24, 48, 72 and 96 hrs. and each sample was analyzed for enzyme activity and specific activity. The enzyme activity and specific activity increased after incubated for 48 hrs. for all isolates into different four media. The isolate A6 strain showed maximum enzyme activity (0.767 U/ml) and specific activity(7.89U/mg) after incubated for 48 hrs. into TGY-Broth with 1% asparagine (Figs. 5 & 6).

Identification of the four bacterial strains

The four isolated bacteria that have ability to produce L-asparaginase, were identified based on morphological, cultural and biochemical tests. A6, C11 and C19were gram positive bacteria with rod shape and endospore forming, catalase – positive, oxidase – negative and starch hydrolysis - positive so there were identified as *Bacillus* species when A11 was gram positive bacteria with cocci arranged in grape like shape and not endospore forming, catalase – positive, oxidase – negative and starch hydrolysis - positive so it was identified as *Staphylococcus* species(Table 3 and Fig. 2).

Table 1: Sites for samples from different locations of Damietta Governorate.

No.	Sample code	Location
1	S	Agriculture sand soil from Gamasa
2	Sc	Agriculture soil from Kafr Shehata
3	Cd	Agriculture soil from Kafr saad
4	A	Bank soil from Nile River
5	C	Bank soil from Ezbet El-Borge canal
6	G	Garden soil from New Damietta Garden

Table 2:Qualitative data of L-asparaginase production by bacteria during primary screening.

No.	Isolates code	Pink color zone	No.	Isolates code	Pink color zone
1	S1	Moderate zone	14	A6	Large zone
2	S2	No zone	15	A10	Small zone
3	S3	Moderate zone	16	A11	Large zone
4	S4	Small zone	17	A18	Small zone
5	S5	Moderate zone	18	A23	Moderate zone
6	Sc6	Very small zone	19	C1	No zone
7	Sc7	Small zone	20	C4	Small zone
8	Sc8	Small zone	21	C5	No zone
9	Cd9	Moderate zone	22	C10	Very small zone
10	Cd10	Very small zone	23	C11	Large zone
11	Cd11	No zone	24	C19	Large zone
12	G1	Small zone	25	C20	No zone
13	G18	Very small zone			

Table 3: Microbiological characteristics of the best L-asparaginase producing bacteria.

Cultural characters on nutrient agar					
Character		A6	A11	C11	C19
Shape		Punctiform	Circular	Punctiform	Circular
Edge		Entire	Entire	Entire	Looped
Elevation		convex	convex	convex	convex
Surface		Smooth	Smooth	Smooth	Smooth
Pigmentation		No pigmented	No pigmented	No pigmented	No pigmented
Optical properties	Color	Creamy	Creamy	Creamy	Creamy
	Opacity	Translucent	Opaque	Translucent	Translucent
Morphological characters					
Gram stain		Positive	Positive	Positive	Positive
Shape		Rod	Cocci	Rod	Rod
Cell diameter (µm)		2.55 µm	1.7 µm	4.08 µm	3.4 µm
Spore stain		Spore forming	Absent	Spore forming	Spore forming
Acid fast stain		Negative	Negative	Negative	Negative
Biochemical characters					
Oxidase test		Negative	Negative	Negative	Negative
Catalase test		Positive	Positive	Positive	Positive
Starch hydrolysis		Positive	Positive	Positive	Positive
Indoleproduction test		Negative	Negative	Negative	Negative
Methyl red test		Negative	Positive	Positive	Negative
Voges-Proskauer test		Positive	Positive	Positive	Positive
Citrate utilization test		Negative	Negative	Positive	Negative
6.5% NaCl		Traces of growth	Growth	Growth	Growth
Lactose fermentation		Negative	Positive	Positive	Negative
Gas production		Negative	Positive	Positive	Negative
Glucose fermentation		Positive	Positive	Positive	Positive
Mannitol fermentation		Positive	Negative	Positive	Positive
Arabinose fermentation		Negative	Negative	Positive	Positive
Maltose fermentation		Negative	Positive	Positive	Positive

Fig. 1:Primary screeningshowing pink color of M9 modified media by C19, A6 and A11.

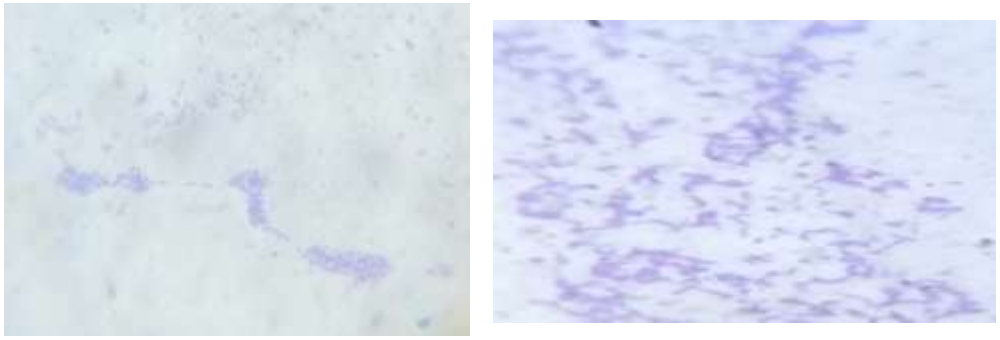


Fig. 2: Gram stain for two L-asparaginase producing bacteria A11 and A6 respectively.

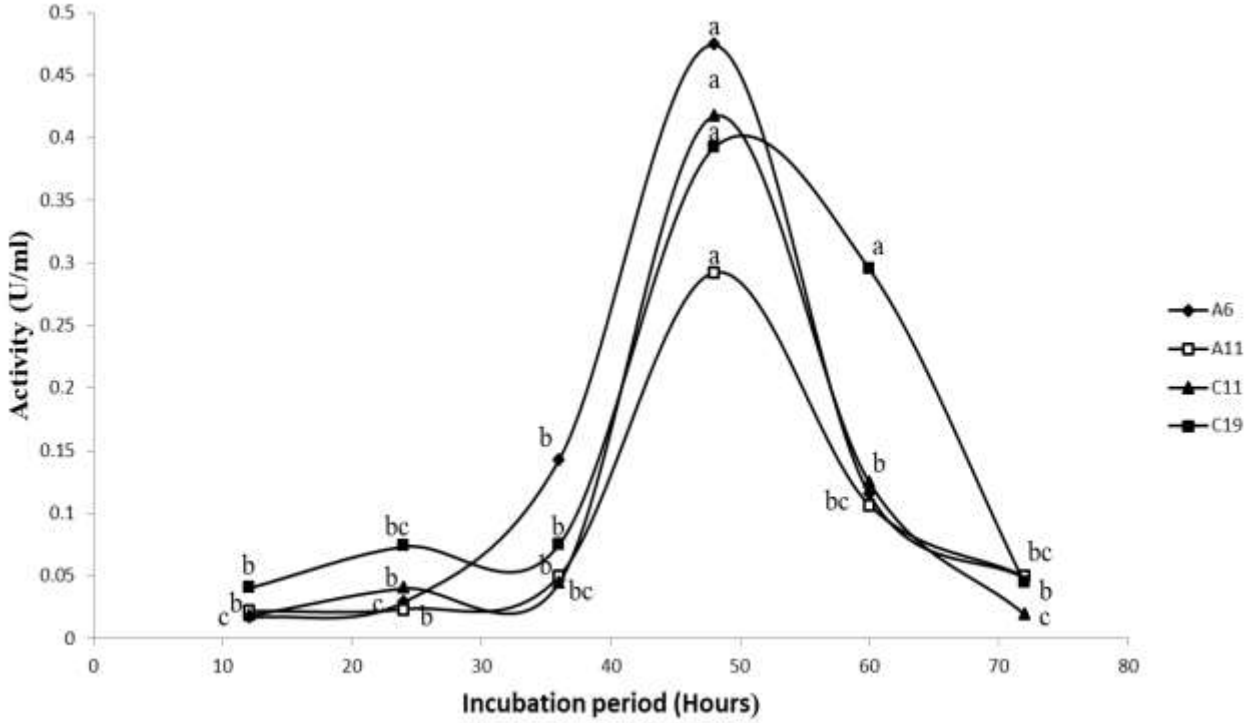
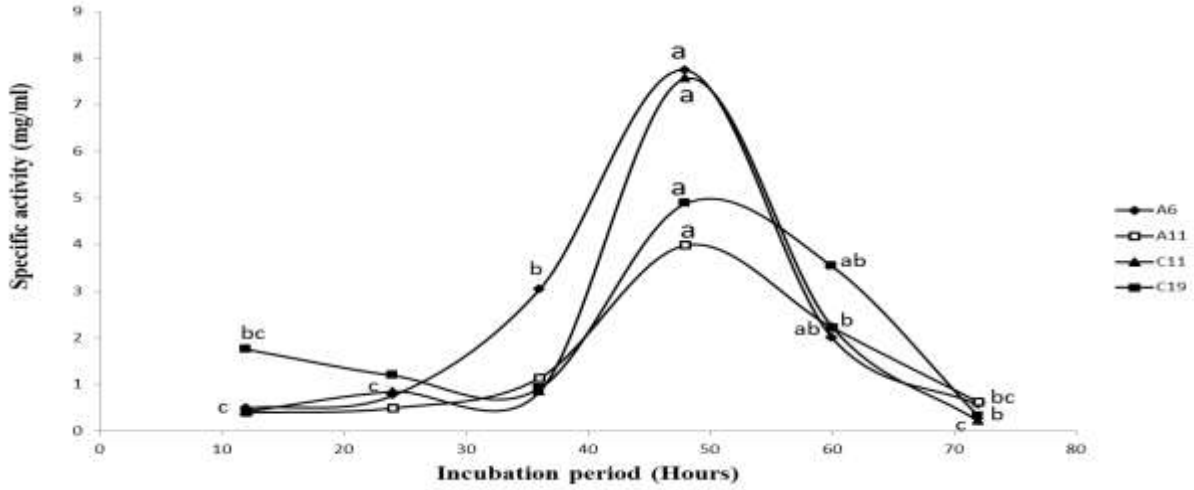


Fig. 3:Activity of L-asparaginase producing bacteria in static fermentation on M9 modified media; different letters indicate different significant values.



Fi.g. 4:Specific activities of L-asparaginase producing bacteria in static fermentation on M9 modified media;different letters indicate different significant values.

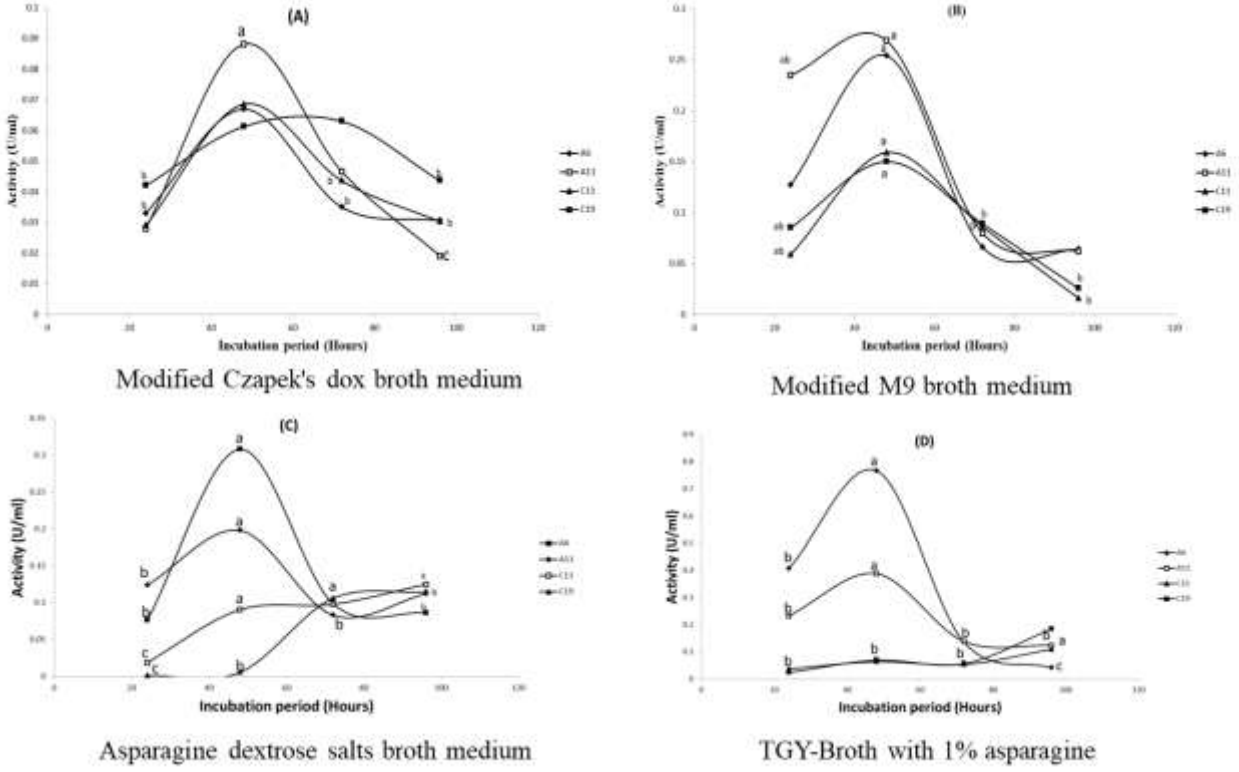


Fig. 5:Activity of L-asparaginase producing bacteria in submerged fermentation on different media; different letters indicate different significant values.

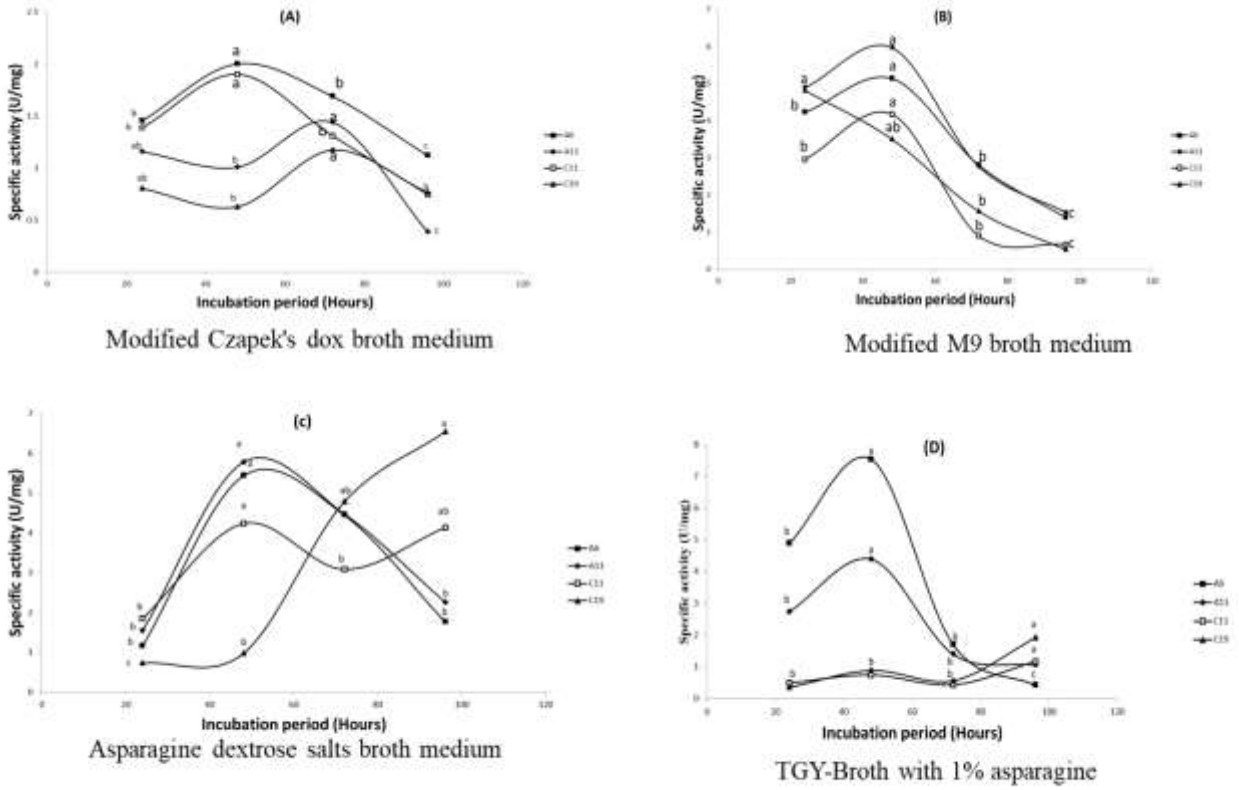


Fig. 6: Specific activities of L-asparaginase producing bacteria in submerged fermentation on different media; different letters indicate different significant values.

DISCUSSION

Recently, L-Asparaginase studying has gained much attention for its anti-carcinogenic potential. Several authors scripted the use of L-asparaginase in cancer therapy (Pieters *et al*., 2011 and Tong *et al*., 2013). In the present study twenty bacterial strains were isolated from six different soil samples have the ability to produce L-asparaginase. This may be attributed to the fact that soil is rich source for potential enzyme producing organisms (Geisseler *et al*., 2010). The result of screening test indicated that L-asparaginase producing bacteria had able to hydrolysis L-asparagine to L-aspartic acid and ammonia that further reacts with water to produce NH_4OH, hence the pH of the medium is basic so the medium color change from yellow to pink. Gulati *et al*. (1997) proved the color transformation was due to L-asparaginase production accordance with this study. The bacterial isolates that had the greatest L-asparaginase activity were identified as *Bacillus* spp. and *Staphylococcus* spp. that is similar to those described by Kamble and Khade (2012) who reported that *Bacillus* spp. were a good asparaginase producing bacteria.The four bacterial isolates (A6, A11, C11 and C19) showed the maximum extracellular L-asparaginase activity with submerged fermentation in tryptone glucose yeast extract with 1% asparagine medium. Similar results were obtained by Sudha *et al*., (2016).

In this study the isolated A6 showed 7.89U/mg of L-asparaginase specific activity with 0.097 mg/ml of a total protein concentration. Kothari and Deshmukh (2014) found 1.79U/mg with 1.33 mg/ml of a total protein concentration. Roberts *et al*. (1968) had achieved 0.950U/mg

of specific activity from *E.coli* HAP strain, when compared to the earlier reports, our study more 50% of specific activity to be increased by using *Bacillus* sp. (A6).

The isolate (A6) showed effective production with the medium containing three nitrogen sources (asparagine, yeast and tryptone) and glucose as sole carbon source at 37˚C and pH 7.0 for 48 hrs. In a previous report, the maximum L-asparaginase activity observed on the media contain peptone as sole nitrogen source and glucose as sole carbon source at 37 ˚C and pH 8.0 for 72 hrs. (Mohapatra *et al*., 1995).

Victoria and Krishnaveni (2010) documented that 10 *Staphylococcal* species showed pink zones around each colony in rapid plate assay on M9 modified medium. That is similar to this study; the isolate (A11) showed large pink zone on modified M9 medium these isolate (A11) was identified as *Staphylococcus* species.

In this study; when different four media were used in screening, we were found the tryptone glucose yeast extract with 1% L-asparagine is the best media for L-asparaginase production, this may by present of three different nitrogen sources and glucose as carbon source which induced the bacterial growth that associated with L-asparaginase production (Hadapsar, 2010).

The four isolates (A6, A11, C11 and C19) have the ability to produce extracellular L-asparaginase on submerged fermentation on tryptone glucose yeast extract with 1% asparagine at 37˚C and pH 7.0 for 48 hrs., this isolates (A6, C11 and C19) were identified as *Bacillus* species, the isolate (A11) was identified as *Staphylococcus* species. These are good preliminary results calling to complete the search on these isolates.

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عزل ومسح عن البكتيريا المنتجة للأسبراجينيز من التربة

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استهدفت هذه الدراسة تقييم قدرة بعض العزلات البكتيريةعلى تحليل الأسبراجين عن طريق إنتاج إنزيم الأسبراجينيز. تم عزل عدد 25 عزلة بكتيرية من عينات تربه تم تجميعها من ستة مواقع مختلفة داخل محافظة دمياط. تم الاختيار المبدئي للعزلات البكتيرية الأكثر نشاطا اعتمادا علي تغير لون أحمر الفينول الموجود في الوسط الغذائي المستخدم لنمو البكتريا من اللون الأصفر إلي اللون الوردي نتيجة تغير وسط التفاعل من الوسط الحامضي الي الوسط القاعدي، وقد دلت النتائج على قدرة 20 عزلةعلى إنتاج الأسبراجينيز، كما تم تقدير ما تنتجه هذه العزلات من الأسبراجينيز بزراعة أفضل أربعة عزلات من حيث إنتاجية الإنزيم علي أربعة أوساط غذائية مختلفة سائلة. تم تعريف أفضل أربعة عزلات من حيث إنتاجية الإنزيم باستخدام الصفات المورفولوجية والخصائص الكيميائية، حيث تم تعريف ثلاثة عزلات على أنهم من عائلة البكتريا العصوية والعزلة الاخرى من عائلة بكتريا المكورات العنقودية.
وقد أوضحت النتائج أن العزلة A6 التي تمتلك القدرة العظمي علي إنتاج الأسبراجينيز من عائلة البكتريا العصوية.