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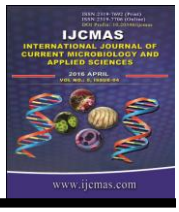

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Determination of L-Glutaminase Activity by Some Bacterial Species

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A B S T R A C T

Out of 200 clinical samples, 178(89%) bacterial isolates were recovered. Based on, cultural, morphological, and biochemical testes, there were 87 (48.88%) isolates of Gram positive cocci belong to the genus *Staphylococcus*, including, 63(35.39%) and 24(13.48%) isolates of *Staph aureus* and *Staph epidermidis* respectively. Whereas the 91(51.12%) remainder isolates were belong to the family *Enterobacteriaceae* and distributed as 56(31.46%), 23(12.92%) and, 12(6.74%) isolates of *E. coli*, *Pseudomonas aeruginosa* and *Citrobacter diversus* respectively. All the bacterial isolates were screened for L-glutaminase enzyme activityusing rapid plate assay. Twenty six (14.61%) isolates were found to be L-glutaminase producers. The zone index was calculated for all L-glutaminase producing samples which are ranged from (3.0-0.25). The maximum zone index was recorded by *Pseudomonas aerogenosa*. The enzymatic activity were ranged from(18.5-6.9)IU/ml. However the maximum activity was recorded for *E. coli*. No.7, Hence this isolate was selected to produce large scale from the L-glutaminase enzyme for further investigations.

Introduction

Microbial enzymes play a major role in the diagnosis, curing, biochemical investigation, and monitoring of many diseases. Microorganisms represent an excellent source of many therapeutic enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. The manufacture of enzymes for use as drugs is an important facet of today's pharmaceutical industry (Saptarshi and Lele, 2011). Biomedical sciences accentuate the involvement of the enzyme L-Glutaminase and other amino acid depleting enzymes as a therapeutic agents for the treatment of tumor (Holcenberg, 1982). L-Glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is a hydrolytic enzyme that deaminates L-glutamine to glutamic acid and ammonia (Roberts *et al*., 1970). Another application of L-glutaminase in food flavoring especially in the soy souse and related industries of the orient.

With the development of biotechnology, microbial glutaminase found newer application in clinical analysis and even in manufacture of metabolites. It uses in biosensors for monitoring glutamine levels

in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid (Sabu *et al*., 2002). Many microorganisms, such as bacteria, yeasts, moulds and filamentous fungi, have been reported to produce L-glutaminase.

This enzyme from microbial origin supposed to be more stable than that from animal and plant sources (Sajitha *et al*., 2013). Commercial production of glutaminase is carried out using submerged fermentation (SmF) technique. Also solid state fermentation (SSF) has emerged as a promising technology for the development of several bioprocesses which include the production of industrial enzymes on a large scale (Athira *et al*., 2014). The main objective of this study is to investigate the production and determined the L-glutaminase activity by some clinical bacterial species.

Materials and Methods

Sample Collection, Isolation, and Identification of Isolates

Two hundred Samples are collected from in and out patients with wound infections admitted to the Sulaimani Teaching Hospital during the period from March 2014 to December 2014. The samples were collected using disposable sterile swabs, they transferred immediately to the laboratories for culturing in Brain heart infusion broth, on Blood agar, Nutrient agar and MacConkey agar, then incubated at 37ºC for 24 hours. There were178 samples yield positive growth. Colonies are purified and used for identification tests. All bacterial isolates were examined by biochemical tests according to Bergey's manual of determinative bacteriology (Holt *et al*., 1994). The results were confirmed by performing Vitek technique. The culture was maintained on Nutrient agar medium slants. Inoculated slants were grown in an incubator at 37 ˚C for 24 hr. After that the slants were stored at 4 ˚C in a refrigerator for short term preservation and sub cultured every 15 days in the abovementioned medium.

Qualitative production of L-glutaminase Enzyme (Screening Test, Rapid Plate Assay)

The minimal agar media (g/l of distilled water) contains NaCl, 0.5; KCl, 0.5; MgSO4.7H2O, 0.5; KH2PO4, 1;FeSO47H2O, 0.1; ZnSO47H2O, 1; L-glutamine, 0.5: as nitrogen source, and supplemented with 2.5% phenol red dye (prepared in ethanol and the pH was adjusted to 7.0). Control plate was maintained without glutamine (instead containing NaNO3 as nitrogen source). After autoclaving, the prepared media were inoculated with 24hr. old bacterial colonies then incubated at 37 ºC for 24 hr. The pink zone around bacterial colonies were observed, and the zone index was calculated according to (Gulati *et al*., 1997).

Zone index = Diameter of zone produced by L-Glutaminase (mm)/ Diameter of bacterial colony (mm).

Inoculum Preparation

The inoculum for all L-glutaminase producing isolates were prepared in 250 ml Erlenmeyer flasks containing 100 ml of above medium at pH 7.0. The medium was autoclaved at 121 ºC (15 lb) for 15 min., then inoculated with the bacterial isolate. The inoculated flasks were kept on a shaker at 150 rpm for 24 hrs, then used as an inoculums.

Quantitative Production of L-glutaminase Enzyme (Large Scale)

The L-Glutaminase production medium

(GPM) was prepared according to Suresh Kumar, *et.al.* (Suresh Kumar *et al*., 2013) with slight modification. The medium composed of (g/l of distilledwater): Galactose 10.0, Yeast extract 10.0, L-Glutamine 10.0, Magnesium sulphate 0.5, KH2PO4 0.5, K2HPO4 0.5, NaCl 10. These components were dissolved and the volume was made up to 1L with D.W. then each 100 ml dispensed in 250 ml Erlenmeyer flasks, autoclaved at121 ºC (15 lb) for 30 min., then they were aseptically inoculated with 3% of the prepared inoculum from Glutaminase producing isolates and incubated at 37 ºC for 24 hrs. at 150 rpm in shaker incubator.

The bacterial cells are harvested in refrigerated centrifuge at 8000 rpm for 20 min at 4 °C. The supernatant was used for enzymatic assay, and the cells washed twice with 0.02M phosphate buffer PH 8. Then the cells were disrupted by ultra sonication (Soniprep 150 sonicator) for 5 min. (intermittent) under cold conditions (Scopes, 1987). The supernatant was the source of crud enzyme and used for further enzymatic assay procedures.

Determination of Enzyme Activity

L-Glutaminase was assayed according to (Imada *et al*., 1973). The reaction mixture, containing 0.5ml of an enzyme preparation,0.5 ml of L-glutamine (0.04 M), 0.5 ml of phosphate buffer 0.1 M (pH 8.0), and 0.5 ml of distilled water to a total volume of 2ml solution was incubated at 37°C for 30 min. The reaction was stopped by addition of 0.5 ml of 1.5 M Trichloro acetic acid. Then to 3.7 ml of distilled water, 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added and color developed was read after keeping the mixture at 20°C for 20 min at 450 nm in a spectrophotometer. Enzyme and substrate blanks were used as controls. One unit of L-Glutaminase activity was defined as the amount of enzyme that liberated 1µmol of ammonia per one minute under optimal assay conditions. Assays were done in triplicate and the mean enzyme activity was expressed as International unit per ml (IU/ml).

Results and Discussion

Isolation and Identification of Bacteria

From 178wound samples which yielded positive growth, there were 87 (48.88%) isolates of Gram positive cocci belong to the genus *Staphylococcus*, including, 63(35.39%) and 24(13.48%) isolates of *Staph. aureus* and *Staph epidermidis* respectively. Whereas the 91(51.12%) remainder isolates were belong to the family *Enterobacteriaceae* and distributed as 56(31.46%), 23(12.92%) and, 12(6.74%) isolates of *E. coli, Pseudo aerogenosa,* and *Citrobacter diversus* respectively as revealed in Table 1. These results depended on morphological characteristics of bacterial isolates on cultural media and Gram staining as well as to the results obtained from conventional biochemical tests as represented in Table 2. and Table 3. The diagnosis of these bacterial species were confirmed by performing Vitek technique.

Qualitative Estimation of L-glutaminase Activity

All the bacterial isolates were submitted to the screening test for producing L-glutaminase enzyme which carried out by rapid plate assay (Gulati *et al*., 1997).

Out of 178 (89%) screened isolates, 26 (14.61%) bacteria were able to form pink zone in plates, Characteristics of L-glutaminase producing bacteria. The bacterial L-glutaminase hydrolysed L-

glutamine to glutamate and ammonia. The acid base indicator dye phenol red converts in to pink colour at basic PH.The zone index was calculated for all L-glutaminase producing samples which are ranged from (3.0 -0.25) as presented in (Table 4).

The maximum zone index was recorded by *Pseudo. aeruginosa*, whereas the minimum zone index was for six isolate of *E.coli.* Three isolates of *Pseudo. aeruginosa* had zone index of 2.75, while zone index of 2 was recorded for four isolates of *Staph aureus*, followed by zone index of 1.75 that recorded for two isolates of *Staph. epidermidis.*

The zone index of 1.5 was recorded for three isolates of *Staph aureus* and two isolates of *Staph. epidermidis.* Where as zone index of 1.25 was recorded for two isolates of *Citrobacter diversus* and zone index of 1 was recorded for one isolate of *Citrobacter diversus.* Ultimately the zone index of 0.5 was recorded for one isolates of *E.coli*.

Many researchers were investigated the production of L-glutaminase from varies microbial origins, including bacteria as *Staph. Aureus, Pseudo. aeruginosa* (Soda *et al*., 1972; Oshima *et al*., 1976; Rashmi *et al*., 2012), *E.coli* (Pruisner *et al*., 1976), yeast and filamentous fungi(Elshafei *et al*., 2014). The production titer value of these enzymes are influenced by microbial strains and fermentation conditions (Iyer and Singhal, 2008).

Quantitative Estimation of L-glutaminase Activity

All the 26 Positive isolates which were screened for L-glutaminase in the above step were further cultured in Glutaminase producing media (GPM) containing L-glutamine as a sole carbon and nitrogen source. Quantitative estimating of L-glutaminase activity by selective isolates was carried out using Nesslerization process. The enzymatic activity were ranged from (18.5-6.9) as shown in table 4.6.

However the maximum activity was recorded for *E. coli.* No.7 despite the narrow zone index that produce by this bacteria in the previous step, this finding might be attributed to intracellular production of the enzyme by this bacteria (Hartman, 1968). Hence this isolate was selected to produce large scale from the enzyme L-glutaminase for further study.

Table.1 Distribution of Bacterial Isolates from Wounds Infections

Source of isolates	***Staph. aureus***	***E. coli***	***P. aeruginosa***	***Staph epidermis***	***Citr. diversus***	**Total isolates**
Infected Wounds	63	56	23	24	12	178
(%)	35.39	31.46	12.92	13.48	6.74	100

Table.2 Type of Tests for *Staph aureus* and *Staph epidermidis* Identifications

Identification tests	*Staph. aureus*	*Staph epidermidis*
Gram stain	+	+
Motile tests	-	_
Catalase test	+	+
Oxidase test	-	_
Mannitol salt fermentation	+	_
Coagulase test	+	+
Type of Haemolysis on bloodagar	**β. Type**	α-type

Table.3 Biochemical Tests for Identification of *E. coli*, *P. aeruginosa* and *C. diversus isolates*

Biochemical test		*E. coli*	*P. aeruginosa*	*C. diversus*
Indole		+	_	+
Methyl red		+	_	+
Voges proskauer		-	_	_
Citrate utilization		-	+	+
Urease production		-	+	V
Oxidase		-	+	_
Catalase		+	+	
Klegller	**Gas production**	+	+	V
	H2S production	-	+	_
	Slope	Acid	Alkaline	Acid
	Bottom	Acid	Alkaline	Acid
Motility		+	+	+

Table.4 Ability of Bacterial Isolates to Production of L- Glutaminase

Isolates species and number. *	Total isolates counts	Isolates No.	Glutaminase production assay (Zone index)
Staphylococcus aureus	63	7, 14, 42, 56	2.0
		15, 27, 51	1.5
Staphylococcus epidermis	24	5, 12	1.75
		1 6, 23	1.5
Pseudomonas aeruginosa	23	15,23	3.0
		1, 9, 20	2.75
Escherichia coli	56	7	0.5
		3, 27, 40,52,22,37	0.25
Citrobacter diversus	12	1, 8	1.25
		10	1.0

* Bacterial isolates numbers that's not mention means not produced L-glutaminase

Table.4-6 L-Glutaminase Activity (IU/Ml) for Bacterial Isolates from Wounds Source

Isolates species and number. *	Isolates No.	Glutaminase activity (IU/ml)
Staphylococcus aureus	7, 14, 42, 56	12.43 to 18.26
	15, 27, 51	8.6 to 10.7
Staphylococcus epidermis	5, 12	11.5, 12.2
	1 6, 23	6.9, 8.5
Pseudomonas aeruginosa	15, 23	12.3, 14.0
	1, 9, 20	8.5 to 11.7
Escherichia coli	7	18.5
	3, 27, 40,52	11.0 to 14.3
	22, 37	7.2, 9.4
Citrobacter diversus	1, 8	10.0, 13.8

*** Bacterial isolates numbers that's not mention means not produced L-glutaminase**

L-glutaminase have been reported in many microbial species but their biochemical, and enzymatic, substrate specificity, molecular weight and antitumor activities vary with genetic nature and cultural conditions which optimized by investigators for various microorganisms as for filamentous fungi by (Nathiya *et al*., 2012).The enzyme activity for *E.coli* estimated by (Hughesd and Williamsodn, 1952), the optimum activity of the L-glutaminase A and B which produce by *E.coli* depends on PH, Glutaminase A have optimal activity at PH about 5, such enzyme would be unsuitable for clinical application where they would be required to be use at PH above 7 as that used by (Roberts *et al*., 1970). Whereas the glutaminase B have maximum activity at pH above 7 (Prusiner, 1975).

The maximum yield of L-glutaminase from *Pseudomonas aeruginosa* and *Serratia marcescens* obtained following optimization of fermentation process by (Rashmi *et al*., 2012). Also L-glutaminase production from aerobic gram positive filamentous bacteria *Streptomyces griseus* under optimized conditionwas reported by (Suresh Kumar *et al*., 2013). With maximum activity of 45IU/ml. However, Tullimilli *et.al.* (Tullimilli *et al*., 2014), reported the maximum activity of L-glutaminase produced by fungal strain *Mucor racemosus* at 969.23 IU/mlafter optimizing culture conditions. It have been concluded from these results that *E.coli* No.7 has potential for large scale production of L-glutaminase enzyme for use in industrial and pharmaceutical applications.

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