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Optimization of Arginine deiminase production from a local higher productive isolate Enterococcus faecium M1

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Abstract:

Arginine deiminase (ADI) is an important enzyme in many biological applications and a cancer treatment agent, recently increasing attention is focused to choose a higher productive strain from different bacterial sources. Eighty nine Enterococcus isolates were obtained from 215 samples (20 clinical UTI specimens, 52 human stool specimens, 6 soil and 11 from sewage water), fifty seven isolates were ADI producer. Twenty five isolates were more active in enzyme production, fourteen of them were identified as E. faecium and 11 isolates were E. faecalis. E. faecium isolate M1 obtained from UTI was the most efficient in ADI production, the specific activity of ADI produced from this isolate was 2.64U/mg protein. The effect of culture medium (Mineral salt broth) components on ADI production and other cultural conditions were determined to find the optimum conditions for enzyme production. The maximum ADI production was achieved when the medium was supplemented with 20mM arginine, 1% sucrose, 1% casein, pH 7.5 and incubated at 37ºC for 18 hours. Under these conditions, the specific activity of ADI was 5.1U/mg protein. By concluding, the present study was designed to select ADI higher productive isolate and optimum culture conditions for increasing the production of this enzyme in order to use it as a potent cancer treatment agent after purifying and characterizing it in the future.

Keywords: *Arginine deiminase, Enterococcus faecium M1, UTI*

Introduction:

L-Arginine deiminase enzyme (EC 3.5.3.6) catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia which is widely expressed in bacteria including Mycoplasma arginini, Pseudomonas aeruginosa and some species of Enterococcus (1).

Production of ADI enzyme is used as routinely test to identify many species of Enterococcus like E. faecium and E. faecalis, but E. pseudoavium, E. raffinosus and E. avium cannot produce this enzyme (2, 3).The uptake of arginine is due to an arginine-ornithine antiport system which does not require metabolic energy (4), whereas the enzyme is essential for the survival of pathogenic protozoa and bacteria (5). E. faecium, formerly classified with fecal streptococci, has been recognized to be of fecal origin (6). However, enterococci are ubiquitous and can be found free-living in soil (7), river and sewage water (2). Enterococci have emerged as important nosocomial pathogens were considered to be low pathogenic and in generally they are not pathogenic in healthy human. E. faecalis and E. faecium caused infections more than other species (8, 9). Among clinical infections disease, the urinary tract infections are the most frequent form of enterococcal disease (10). Arginine deiminase enzyme has not been studied in a sufficient manner with other species of Enterococcus, thus the aims of this study are to isolate arginine deiminase producing Enterococcus spp. from different environmental sources to choose the high productive strain and optimize culture conditions, to obtain a high yield of the enzyme which can be used in the future as a therapeutic agent for cancer treatment.

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Materials and Methods:

Sample collection: Healthy human stool specimens 68 samples and 103 urine samples from patients with UTI, also 25 soil and 19 sewage water samples were locally collected in sterile containers.

Isolation and identification of Enterococcus: The collected samples were inoculated on (Ej) medium (11) modified by (12). The bacterial colonies were transferred to the selective and differential medium azid bile esculin agar and incubated at 37◦C, the brownish colonies were selected, purified and identified (13, 14).

Semi-quantitative screening for ADI production: Enterococcus isolates were semi-quantitatively screened for arginine deiminase production on Mineral salt agar with 2gm/L arginine (15) instead of glutamine and arginine broth medium (16, 17).

Quantitative screening assay of arginine deiminase activity in intact cells:

The enriched isolates were quantitatively screened for ADI production, 0.2ml of McFarland calculated culture cell suspension was transferred to 4.8ml of mineral salt broth containing (gm/L) 2gm arginine, 0.5gm MgSO4.7H2O and 1gm K2HPO4, pH was adjusted to 7.0 and incubated at 37ºC for 24h. The cells were precipitated by centrifugation at 6000 rpm for 30 minute, washed with 0.02 M phosphate buffer pH 7.0 and resuspended in the same buffer at a final concentration of 0.5mg/ml of bacterial cells. Arginine deiminase activity was assayed after drawing the standard curve of citrulline (enzyme product) concentrations in the reaction mixture (1ml) containing 0.4ml of 50mM phosphate buffer (pH 7.0), 0.4ml L-arginine (20 mM and 0.2ml cell suspension and incubated at 37ºC for 15min., the reaction was stopped by the addition of 10% trichloroacetic acid, and centrifuged at 6000 rpm for 30 min. The citrulline formed was measured by the modified Archibald method (18), 1.4ml of acid mixture (18 M H2SO4-14 M H3P04; 1:3,vol/ vol) was added to 1 ml of the supernatent after centrifugation of trichloroacetic acid-treated sample, and 0.5 ml of 3% diacetylmonoxime were added, mixed together, and boiled in the dark for 15min then cooled in the dark for 10 min, the absorbance was measured at 490 nm and the enzyme activity was estimated according to the standard curve of citrulline.

Enzyme activity (unit) is defined as the amount of enzyme that liberates 1 micromole of citrulline in one minute at the assay conditions.

Assay of protein concentration:

Protein was assayed by Lowry method (19) after drawing the standard curve of citrulline (enzyme product) concentrations and the standered curve of bovine serum albumin (protein) concentrations..

Determination of the optimal conditions for arginine deiminase production: The optimal conditions were determined using (Mineral salt broth medium), included:

Arginine concentration: Different concentrations of arginine were used (10-70 milimolar/ml).

Carbon source: Different carbon sources were used at 1% concentration included: (glucose , fructose , maltose , sucrose , arabinose , rhamnose , xylose, sorbitol and manitol) with arginine.

Nitrogen source: the production medium was supplemented with 1% of different nitrogen sources (meat extract, peptone, trypton, yeast extract, casein, NH4Cl, lysine, histidine , (NH4)2SO4 and NaNO3) with and without arginine.

Incubation period: Different periods of incubation (6-48 hrs) were applied.

pH: the production medium was prepared at different pH ranging between 5.0-9.0.

Incubation temperature: bacterial culture was incubated at (25-50ºC).

Results:

Isolation of *Enterococcus*

Table1 describes the source, number and percentage of Enterococcus isolates obtained from different samples and the percentage of these isolates were (58.4%) from stool samples, 22.4% from UTI samples, (12.3%) from sewage samples and 6.74% were from soil samples of total isolates.

Table 1: Source and number of enterococcal isolates.

Isolation Sources	No. of samples	No.of *Enterococcus* isolates	*Enterococcus* percentage from each source %	*Enterococcus* percentage out of total isolates
UTI	103	20	19.4	22.4
Stool	68	52	76.4	58.4
Soil	25	6	24	6.74
Sewage	19	11	57.8	12.3
Total	215	89	41.3	100

Screening for arginine deiminase production from *Enterococcus* isolates

Semi-quantitative screening for ADI presented that during 48 hrs. of incubation, enterococcal isolates varied in growth on mineral salt agar medium, 25 isolates were observed during 24hr and 32 isolates appeared during 48hr, while 32 isolates couldn't grow. The arginine hydrolyzing (57 isolates) varied in color intensity resulted from reaction of Nessler's reagent, 25 isolates revealed orange color then converted to red precipitate, while the others formed pale yellowish color converted to orange or yellow precipitate after few days of incubation at room temperature. These results presented in table 2.

Identification of *Enterococcus* species: Some biochemical tests were run to identify the species of arginine deiminase producing bacterial isolates, the results revealed that these isolates are belonged to two species ,14 isolates (56%) were belong to E. faecium and 11 isolates (44%) were belong to E. faecalis.

Quantitative screenings of *Enterococcus* isolates: The specific activity (S.A) of ADI enzyme was measured for 25 isolates, E. faecium M1 isolated from UTI revealed higher specific activity (2.64 U/mg protein) than the other isolates (Table 2) thus it was selected for the following steps.

Effect of culture medium components on arginine deiminase production Arginine concentration: Media with different concentrations of arginine were used; 20mM of arginine was the most suitable concentration, the specific activity reached to 3.1U/mg protein (figure1).

Carbon source: figure (2) indicated that a maximum production of ADI was achieved in medium containing sucrose with arginine, ADI specific activity reached to 3.9 U/mg. Other carbon sources showed lower enzyme activity.

Table 2: Productivity of ADI for 25 Enterococcus isolates cultured in arginine broth medium incubated at 37ºCfor 96hrs and their specific activities on mineral salt broth medium with arginine (+++: Orange, ++:Dark yellow,+:pale yellow ,- :negative).

Enterococcus species, and source of isolation	Specific activity U/mg	Intensity of the color	*Enterococcus* species and source of isolation	Specific activity U/mg	Intensity of the Color
***E. faecium*(M1),UTI**	**2.64**	+++	***E.faecalis*(M14), stool**	**1.38**	++
***E. faecium*(M2), UTI**	**2.58**	+++	***E.faecium*(M15),UTI**	**1.25**	++
***E.faecium*(M3),sewage**	**2.53**	+++	***E.faecium*(M16),stool**	**1.21**	++
***E. faecalis*(M4), UTI**	**2.41**	+++	***E.faecalis*(M17)sewag**	**1.13**	++
***E. faecium*(M5), stool**	**2.28**	+++	***E. faecalis*(M18)stool**	**1.1**	++
***E.faecalis*(M6),sewage**	**2.22**	+++	***E.faecium* (M19)stool**	**1.07**	++
***E. faecalis*(M7), UTI**	**2.13**	+++	***E.faecium*M20sewage**	**0.98**	+
***E.faecium*(M8), UTI**	**2.06**	+++	***E. faecalis*(M21),stool**	**0.98**	+
***E.faecium*(M9), stool**	**2**	+++	***E. faecalis*(M22),stool**	**0.91**	+
***E.faecium*(M10), UTI**	**1.96**	+++	***E.faecium* (M23)stool**	**0.8**	+
***E.faecalis*(M11),sewage**	**1.88**	++	***E.faecium* (M24)stool**	**0.73**	+
***E.faecalis*(M12), UTI**	**1.6**	++	***E.faecium* (M25)stool**	**0.66**	+
***E.faecalis*(M13),UTI**	**1.52**	++	-	-	-

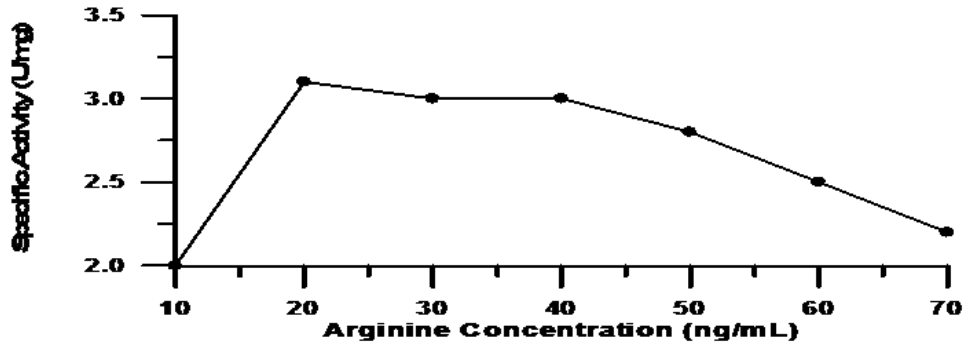

Figure (1) ADI production by *E. faecium* M1 cultured in mineral salt broth medium containing different concentrations of arginine

Nitrogen source: The maximum production of enzyme was achieved when the production medium containing arginine was supplemented with 1% casein, figure (3), the specific activity reached to 4.5U/mg. Medium with casein and without arginine also gave a good result for ADI, the specific activity in this medium was 3.3U/mg. Medium supplemented with casein and sucrose with (20Mm) arginine was used for production of ADI in the following steps.

Incubation period: ADI production initiated during the first 6 hours of incubation (Figure 4), the specific activity reached the maximum value (4.8U/mg) after 18 hours of incubation then decreased to (0.9U/mg) after 48h of incubation.

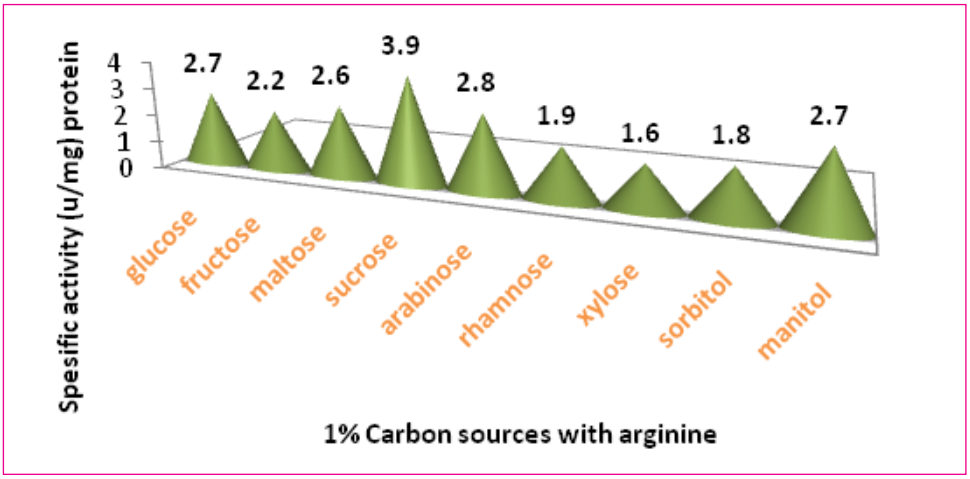

Figure 2: Effect of different carbon sources with arginine on ADI production after incubation for 18 hrs. at 37ºC.

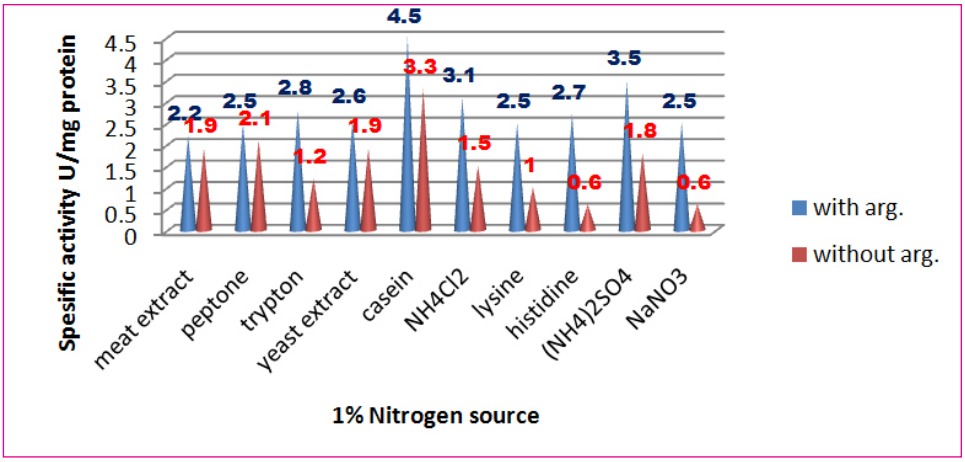

Figure 3: Effect of (1%) different nitrogen sources on ADI production with and without arginine after incubation for 18 hrs. at 37ºC

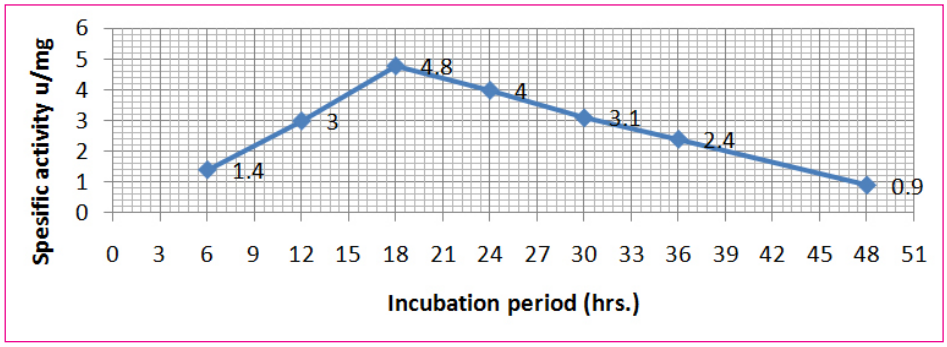

Figure (4): Effect of incubation period on ADI production by *E. faecium* M1 at 37ºC

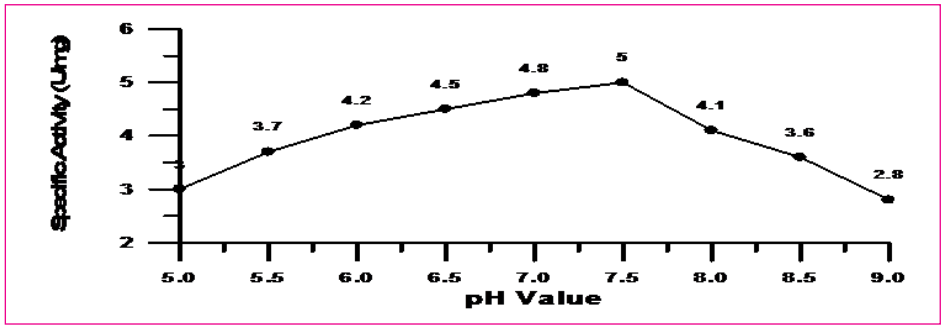

Figure (5): Effect of medium pH on ADI production by *E. faecium* M1 for 18hrs. of incubation period at 37ºC.

Effect of pH: The results in figure (5) showed that ADI exhibited different activities at pH (5.0-9.0). The maximum ADI production was obtained when the pH value of the production medium was 7.5 with S.A of 5U/mg protein.

Effect of incubation temperature: Different incubation temperatures (25-50ºC) were adopted to determine the optimum one for ADI production by E. faecium M1.

Maximum production of ADI was at 37ºC, the specific activity was 5.1U/mg at this temperature. The enzyme activity decreased in other temperatures but not diminished (figure 6).

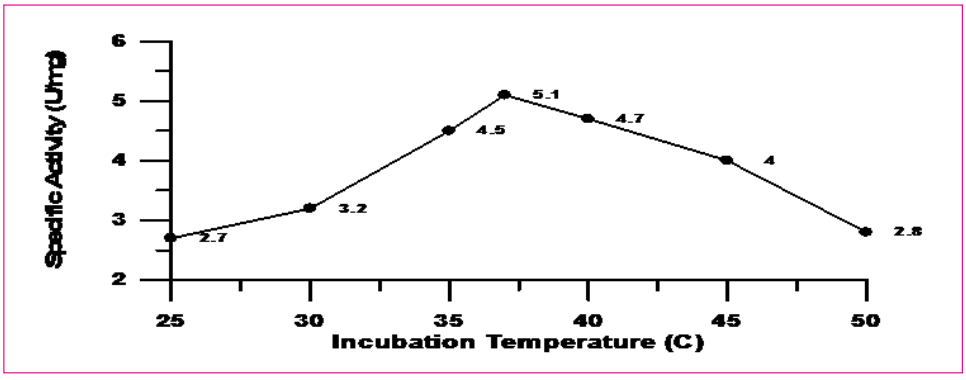

Figure 6: Effect of incubation temperature on ADI production by *E. faecium* M1

Discussion:

Most enterococcal isolates were from stool samples (58.4%), this may be due to the natural inhabitance and usual ecological niche for this genus is human and other animal intestines. The percentage of UTI isolates was 22.4% because enterococci is an emerging nosocomial pathogen possess a number of virulence factors that permit adherence to host cells and facilitate tissue invasion (20). Sewage samples had (12.3%) of total isolates, this genus could be indicative of sewage contamination and can be easily isolated and confirmed by a relatively simple bacteriological technique (2), the lower number (6.74%) of isolates was that of soil, this mean that soil is contaminated with this bacteria, which indicate that populations of enterococci may be a part of the natural soil microflora, the extent of Enterococcus occurrence and distribution in

a wide range of soils remained unknown (7).

Semi-quantitative screening presented that, mineral salt agar medium was very suitable method in screening for the production of ADI because it had arginine as a sole energy source, thus the growing isolates confirmed that they produced this enzyme, depending on, these results indicated that ADI was produced by most enterococcal isolates and some of them 25 isolate were more active ADI producers. Some species of Enterococcus cannot produce ADI like E. pseudoavium, E. raffinosum, and E. avium (3). The results of secondary screening step were compatible with the first step. The arginine hydrolyzing isolates varied in intensity and diameter of precipitated colour (Table 2), which indicated the production of ammonia as a final product of ADI reaction and other two enzymes (ornithine transcarbamylase and carbamate kinase) .The color intensity indicates bacterial activity to hydrolyze arginine and liberate ammonia.

E. faecium M1 isolated from UTI revealed maximum ADI production. This result demonstrates that the pathogenic strains may have the ability to produce ADI enzyme with high activity in order to produce ATP and to survive in infection position which considered a non-natural habitat for this microorganism (5).

Effect of culture medium components on arginine deiminase production.

Arginine concentration: 20mM arginine was the most suitable concentration, for ADI production, this may prove that arginine induces the production of ADI at low level concentrations more than the high levels. The expression of the arc operon in Lactobacillus sakei is induced by arginine (21), some authors (1) reported that the optimum concentration of arginine in culture medium was 50 mM/L during growing of
E. faecalis.

Effect of carbon source: The maximum production of ADI was achieved when the combination of arginine and sucrose were incorporated in medium. Addition of arginine to growing cells resulted in the coinduction of ADI but growth on glucose-arginine decrease the specific activity of the arginine fermentation system (1).

Effect of nitrogen source: The results revealed that the production of E. faecium M1 ADI varied according to the type of nitrogen source. Casein supported ADI production more than other nitrogen sources which reflects the efficiency of this nitrogen source (in the combination with arginine) to provide growth requirements and production of ADI, due to its composition as a protein, supplies essential amino acids for construction of many important compounds in the cells included enzymes. Ammonium sulfate followed casein in supporting ADI production. It was found (22) that peptide is the best nitrogen source for production of enzyme from E. faecalis NJ402.

Effect of incubation period: ADI production initiated during the first 6 hours of incubation with gradual increase in productivity with increasing of incubation period.

Enzyme specific activity reached the maximum value after 18 hours of incubation (in exponential growth phase) then decreased after 48h of incubation. Other related studies found that higher enzyme production occurred when Lactobacillus sakei CTC 494 culture was in the mid-exponential growth phase and the highest relative gene expression level was in the end-exponential growth phase of L. sakei K strain (21).

Effect of pH: Maximum ADI production was obtained when the pH value of the production medium was 7.5, this may attributed to that this bacteria was isolated from human body that has neutral pH thus it was survived at this pH. The pH of production medium affects the enzyme activity by impact the solubility of nutrients and transition of them through cell membrane and its effect on ionic state of the substrates and stability of enzymes then interacted with the growth of bacteria and production of enzymes (23).

Effect of incubation temperature: The results indicated that ADI is produced at a wide range of temperatures, Maximum production of ADI was at 37ºC; other temperatures decreased the enzyme productivity but not diminished it. this may attributed to that this isolate favores this temperature for growth and metabolism, since the normal habitat of it (human) thus it is adapted to survive at 37 ºC.

Furthermore this isolate tolerates higher temperatures and can grow and produce the enzyme at 50ºC but with lower production than 37ºC. The maximum ADI production from E. faecalis NJ402 was obtained when the incubation temperature was 37ºC (22).

Conclusions:

From the results of this study, the following conclusions can be made:-

1. The most active arginine deiminase productive strain was E. faecium M1 Isolated from (clinical) urinary tract infection source.
2. Semi-quantitative screening presented that, mineral salt agar and arginine broth media were very suitable methods in screening for ADI production and selecting enzyme active isolates.
3. The optimum condition for ADI production was achieved when the medium was supplemented with 20mM arginine, 1% sucrose, 1% casein, pH 7.5 and incubated at 37ºC for 18 hours.

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الظروف المثلى لأنتاج أنزيم Arginine deiminase من العزله المحليه الأعلى أنتاجا Enterococcus faecium M1

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الخلاصه:

يعتبر أنزيم Arginine deiminase من الأنزيمات المهمه لدوره القوي والفعال في علاج مرض السرطان الفتاك أضافة الى استخدامه في تطبيقات علاجيه أخرى، لذا تهدف هذه الدراسه الى عزل السلاله البكتيريه الأكفأ أنتاجا للانزيم من جنس Enterococcus ومحاولة أيجاد الظروف المثلى لأقصى أنتاج للأنزي. تم الحصول على 89 عزله لبكتريا Enterococcus(20 عزله من عينات التهاب المجاري البوليه، 52 من عينات خروج الأنسان،11 من مياه المجاري و6 عزلات من التربه) وكانت 57 عزله منها منتجه لأنزيمArginine deiminase. أظهرت 25 عزله نشاطا أكثرلأنتاج الأنزيم لذا تم تشخيصها الى أنواعها وكانت 14 عزله منها تابعه للنوعE. faecium و11 عزله تابعه للنوع E. faecalis وقد تم اختيار العزله الاعلى أنتاجا للانزيم والتابعه للنوع E. faecium من أصابات المجاري البوليه وأبدت اقصى فعاليه للأنزيم بمقدار 2.64 وحدة/ملغم بروتين. تمت دراسة الظروف المثلى لأنتاج الأنزيم على الوسط الملحي السائل ولوحظ أن أقصى أنتاجيه للأنزيم تكون عند استخدام الأرجنين بكمية 20 ملي مولر و1% من السكروز و1% من الكازائين برقم هيدروجيني 7.5وحضنها بدرجة حراره 37م° لمدة 18 ساعه حيث ازدادت الفعالية الأنزيميه الى 5.1 وحده/ملغم بروتين.أن ارتفاع الفعاليه الأنزيميه بمقدار الضعف باستخدام الظروف الزرعيه المثلى يشجعنا مستقبلا على استخدام هذه العزله الكفوئه والظروف المذكوره لأنتاج الأنزيم صناعيا واستخدامه في التطبيقات العلاجيه بعد تنقيته ودراسة خواصه.