مجلة كلية التربية الاساسية
كلية التربية الاساسية – الجامعة المستنصرية
Journal of the College of Basic Education Vol.32 (NO. 136) 2026, pp. 462-476

Isolation of a Novel *Brevibacillus parabrevis* PS12 Strain from Soil and Determination of Optimal Conditions for Arginase Production

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Abstract:

Soil-occupy *Brevibacillus parabrevius* PS12 species are known for their metabolically versatile and it is being discovered as source of natural enzymes valuable to industry. Arginase catalyzes the processing of L-arginine to L-ornithine and urea, it is the one that plays a significant role in nitrogen metabolism Finding novel bacterial strains from environmental sources that are capable of producing the arginase enzyme is crucial for expanding microbial resources for industrial and pharmaceutical use. Among the 20 isolates, one isolate was identified as *Brevibacillus parabrevis* PS12 (97% 16S rRNA similarity, GenBank accession no. (PX118439.1), **representing the first report from Iraqi soil**. Arginase activity reached 8.3 U/mg protein a peak in nutritional source with maltose as a arbone source and casein as a Nitrogen source, the physical parameters also effect on enzyme specific activity at 7.0 pH, 37°C, and 48 hours of incubation time with shaker incubator 150 rpm to ensure the homogeneity of all medium components and continuous aeration all these factors contributed to an increase in bacterial biomass which led to a rise in the specific activity of arginase.

Keywords*: Brevibacillus parabrevis* PS12, Arginase, Optimal conditions, Modified Arginine Media, 16S rRNA sequencing.

Introduction

The taxonomic group of *Brevibacillus* re-categorized from Bacillus based on morphological and molecular characteristics (16 S rRNA detection) Placed in family Paenibacillaceae (Firmicutes) and revealed evolutionary branches within order Bacillales (Dobrzyński *et al*., 2023). Members of this genus exhibit metabolic elasticity, form endospores, and can be adaptive for diverse conditions, including soil, water, and industrial environments, which allows them to tolerate a wide range of environmental conditions (Harirchi *et al*., 2022). *Brevibacillus* species is not disease-causing, produces bioactive such as antimicrobial agents and biological surfactants (Saxena, 2015), and

produces multiple of extracellular enzymes include, proteases, amylases, cellulases, lipases, and oxidoreductases, rendering them useful for industrial, pharmaceutical, and biotechnological applications (De Giani *et al*., 2021; Deng *et al*., 2023). Although numerous studies have emphasized the genus's role in producing industrially relevant enzymes, the potential of arginase remains insufficiently explored, representing a critical gap in microbial research (Liu *et al*., 2024؛ Akinsemolu *et al*., 2024). Enzymes are environmentally friendly biocatalysts from renewable biological resources. Microbes are used to produce enzymes because they grow quickly and are easy to use on a large scale, and researchers are looking for new enzymes that are stronger and more stable (Sheldon & Brady, 2021؛ Elazzazy *et al*., 2025). arginase is an enzyme that helps convert L-arginine into L-ornithine and urea, important for the body's metabolism, it's also affecting the immune system, cell growth, and collagen production, and may help treat diseases like cancer, high arginine levels, brain disorders, autoimmune diseases, and asthma (Barzkar *et al*., 2025). Studies indicate that this enzyme has promising therapeutic potential against various tumors, hyperargininemia, neurodegenerative disorders, autoimmune diseases, and allergic asthma (Clemente *et al*., 2020؛ Maggi & Scotti, 2019). This study aimed to isolate a novel *Brevibacillus parabrevis* strain (PS12) from soil in Iraq and optimize the best conditions to produce arginase for possible medical use.

Materials and Methods

Thirty animal sheds soil samples were cultured on Nutrient, MacConkey, and De Man, Rogosa, and Sharpe (MRS) agars, with isolates identified via biochemical tests, Vitek-2, and 16S rRNA gene sequencing for unclassified isolate, while nutritional and physical condition were optimized to increase enzyme yield.

Bacterial isolation and identification

Arginase-producing bacteria were isolated from animal sheds soil using the serial dilution method and cultured on selective media (Nutrient and MacConkey agar, and on MRS medium) with 0.05g/L cycloheximide as antifungal.

Bacterial identification

Morphological

Colonies were investigated for morphology, including surface characteristics, color and elevation. Microscopic examination was performed to identify cell shape, arrangement, and Gram stain reaction (Rocha *et al*., 2023).

Biochemical test

Biochemical tests were carried out to characterize the bacterial isolates, including tests for catalase, citrate, gelatinase, and urease. For the catalase test, bacterial colonies were mixed with 30% water, and the appearance of bubbles indicated a positive reaction. Citrates were evaluated using Simmons citrate agar, where growth and a change in color from green to blue confirmed a positive result. Gelatinase production was determined using gelatin agar, where the liquefaction of the medium indicated enzyme activity. Urease activity was tested in urea broth, where the appearance of a light pink (fuchsia) color indicated a positive reaction (Ray *et al*., 2020) .

Molecular identification

Molecular identification via 16S rRNA sequencing using universal primers (27F: AGAGTTTGATCCTGGCTCAG; 1492R: TACGGCTACCTTGTTACGACTT) under the thermal cycling conditions listed in (Table 1). The selected strain was submitted to GenBank and used for further optimization of arginase production.

Table 1. Thermal cycling conditions

Cycle step	Temperature °C	Time	Cycles
Initial Denaturation	95	1 min	1
Denaturation	95	30 sec	40
Annealing	60	30sec	
Extension	68	1 min	
Final extension	68	5 min	1

Qualitative and Quantitative methods for arginase

Qualitative detection was performed on Modified Arginine Medium g/L (glucose 5, yeast extract 5, peptone 5, K_2 HPO_4 1, L-arginine 10, agar 20, few drop phenol red) and incubated at 30°C for 24 hur. Quantitative estimation was carried out in Enrichment Modified Arginine agar medium g/L (glucose 5, yeast extract 5, peptone 5, K_2 HPO_4 1, L-arginine 10) by incubating submerged cultures at 30°C, 150 rpm for 48 h to measure enzyme production levels (Zhang *et al*., 2013).

Enzyme activity assay

The reaction mixture contained 0.2 ml phosphate buffer (pH 7.0), 0.5 ml crude enzyme, and 0.1 ml $MnCl_2$, pre-incubated at 37°C for 10 min. The reaction started with 0.1 ml L-arginine and continued for 30 min at 37°C,

then stopped with 1 ml perchloric acid. Add 1ml from sample aliquot to mixed color and mixed acid reagents after that covered firmly and placed in boiling water bath for 20 min, cooled at room temperature and OD were measured at 520 nm to quantify arginase activity from urea liberated (Langenfeld *et al*., 2021). One unit of enzyme activity is defined as the amount of enzyme that liberates 1µM of urea at 37℃, using L-arginine as substrate.

Optimization of Factors Affecting Arginase Production

Various carbon sources (glucose, dextrose, glycerol, sucrose, maltose) and nitrogen sources (peptone, ammonium sulfate, tryptone, yeast extract, casein) were tested individually at a concentration of 0.5% (w/v), with varying temperatures (27, 32, 37, 42, 47°C), incubation periods (24, 48, 72, 96 h), and pH levels (4, 5, 6, 7, 8, 9) in the enrichment/production medium, to evaluate their effects on arginase activity, protein content, and specific activity. Enzyme extraction and activity measurement were carried out depending on Langenfeld method (Langenfeld *et al*., 2021).

Statistical Analysis

The enzyme activity (U/ml) was calculated using the formula: {enzyme activity (U/ml) = µmoles of urea released /Time of enzyme action x Volume enzyme(ml)}. The specific activity (U/mg) was calculated using the formula: {Specific activity (U/mg protein) = Enzyme activity (U/ml) / Protein concentration (mg/ml)}. The curves of the tested parameters were generated using Excel to illustrate the variations in enzyme production.

Results and Discussion

Bacterial isolation and identification

Of the 30 soil samples, 20 bacterial isolates were isolated. Nineteen (95%) of these isolates were identified using Vitek-2 and conventional methods, while one (5%) isolate was not identified by Vitek-2. The identified isolates were *Escherichia coli* 5 (25%), *Klebsiella pneumonia* 2 (10%), *Kocuria varians* 2 (10%), *Leconostoc mesenteroides* 3 (15%), *Psedomonas aeruginosa* 5 (25%), and *Klebsiella pneumonia* 2 (25%). The unknown isolate was identified by 16S rRNA gene sequencing. Soil bacteria are an important source of enzymes used in food, medicine, and environmental cleanup, and species of *Brevibacillus* have role in biofertilizers in agriculture. Finding strain PS12 in Iraqi soil shows its importance and that local soils are a good source for discovering new useful bacteria (Khalil & Hasan, 2024؛ Yahya *et al*., 2021) .

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Molecular analysis

Identified of 16S rRNA gene

The 16S rRNA gene amplicons from the unknown isolate were visualized on a 1% agarose gel stained with ethidium bromide, showing single bands of ~1400 bp, compared with ladder 100-2000 bp, matching the expected full-length 16S rRNA gene size, (Figure 1).

Sequencing of 16S rRNA gene

The 16S rRNA gene of the bacterial isolates was sequenced using the Sanger method on an ABI 3730xl instrument, and the data were analyzed with Geneious software to accurately identify the isolates. The results of 16S rRNA gene sequencing demonstrated that the 1427 bp sequence showed 97% identity with *Brevibacillus parabrevis* in BLASTn analysis (bit score 1910, E-value 0.0, 0% gaps) and was deposited in GenBank (PX118439.1), and the isolates named as *Brevibacillus parabrevis* strain PS12. Phylogenetic analysis tree designed by MEGA X software and placed PS12 in a well-supported clade within the *Brevibacillus parabrevis* group, closely related to strains from India, China, Pakistan, and Egypt, with 94% bootstrap support, suggesting possible regional genetic divergence, (figure2). Phylogenetic analysis positioned *Brevibacillus parabrevis* PS12 (H250806-020 E17 PS12 IRAQ) on a separate sub-branch from reference strains from India, China, Pakistan, and Egypt, marking it as the first characterized *B. parabrevis* isolate from Iraqi soil. This finding is in line with previous reports of novel *Brevibacillus* species isolated from soil, such as *Brevibacillus ginsengisoli* Gsoil 3088^T (Baek *et al*., 2006), and *Brevibacillus halotolerans* LAM0312^T (Song *et al*., 2017), highlighting the genus's ongoing expansion and the potential for discovering genetically distinct strains in diverse environments.

Morphological and biochemical tests of *Brevibacillus parabrevis PS12* strain

The *Brevibacillus parabrevis* PS12 strain appeared as Smooth, flat, creamy to yellow in color on MRS medium, as shown in (figure 3), and appeared as Gram-positive, rod-shaped, occur in singly, pairs, and chains under light microscope. The color may result from carotenoid or flavin, which Preserve the cells from oxidative damage and ultraviolet radiation (Lee *et al*., 2025). In addition, the microscopic examination showed Gram-positive, rod-shaped cells Appearing singly, in pairs, or short chains. These Traits confirm the identification of strain PS12 as *Brevibacillus parabrevis* (Ray *et al*., 2020).

The biochemical tests showed that the Catalase and Gelatinase tests were positive, and Citrate and Urease tests were negative, (Table 2). In contrast, the bacteria were negative for citrate and urease, indicating that this strain cannot utilize citrate as its sole carbon source, cannot hydrolyze urea to obtain nitrogen source (Khatoon *et al*., 2022؛ Dela Cruz & Torres, 2012). These biochemical results are consistent with the general characteristics of the genus *Brevibacillus* species. These General characteristics of the genus *Brevibacillus* are partially consistent with studies on *Brevibacillus* species published by (Ray *et al.*, 2020).

Table 2. The biochemical test of *Brevibacillus parabrevis*

Test	Result
Catalase	Positive
Gelatinase	Positive
Citrate	Negative
Urease	Negative

Qualitative and Quantitative Assessment of Arginase

Arginase production was assessed by culturing the *Brevibacillus parabrevis* PS12 isolate on Modified Arginine Medium (MAM) supplemented with phenol red at 30 °C for 24hr. The results showed that the isolate formed pink/red zones around the colonies, indicating positive arginase activity (figure 4). Quantitative analysis revealed that *Brevibacillus parabrevis* PS12 exhibited the highest specific activity (0.380 U/mg protein), demonstrating its strong potential for enzyme production.

Optimization of Factors Affecting Arginase Production

The results obtained from our study illustrated that the production of arginase by *Brevibacillus parabrevis* PS12 was greatly affected by both nutritional and physical parameters. For nitrogen sources, casein supported the highest enzyme specific activity, reaching 8 U/mg protein. Yeast extract and tryptone also supported enzyme production but to a less markedly, giving specific activities of 5 U/mg protein and 4.3 U/mg protein, respectively. On other hand, ammonium sulfate and peptone showed the lowest effect among the tested nitrogen sources, with activities of 4.1 U/mg protein and 4.0 U/mg protein (Figure 5-A). Our result disagreement to findings by (Nadaf & Vedamurthy, 2020), who reported yeast extract as optimal nitrogen source for *Pseudomonas aeruginosa*, and maximum arginase activity with yeast extract for *Klebsiella pneumoniae* N1 reported by (Alameedy & Jebor, 2024), and maximum arginase activity with peptone for *Alcaligenes aquatilis* BC2 reported by (Assega *et al*., 2025).

. Our results show that the carbon source from maltose was established to be the most effective, leading to 4 U/mg protein of enzyme specific activity, succeeded by sucrose at 3.3 U/mg protein. In contrast, dextrose, glucose, and glycerol resulted in much lower enzyme production, with activities of 1.3, 1.0, and 0.66 U/mg protein , respectively (Figure 5-B). These results are consistent with previous studies suggesting that disaccharides provide both energy and carbon skeletons more efficiently, enhancing enzyme production (Selim *et al*., 2024); Our study Corresponds with previous studies, showing analogous maltose effects on arginase activity as reported for *Pseudomonas aeruginosa* (24) and *Alcaligenes aquatilis* BC2 (Assega *et al*., 2025).

The results of the physical parameters indicated that arginase activity was significantly affected by pH, showing maximum production at a neutral pH of 7.0, while both acidic and alkaline conditions led to a marked decline in enzyme activity (Figure 5-C). The observed optimal pH disagree with *Pseudomonas aeruginosa* pH 8.5 (Baek *et al*., 2006), *Bacillus licheniformis* OF2 pH 8.0 (Selim *et al*., 2024), and *H. pylori* pH 6.1 (McGee *et al*., 2004).

In the present study, temperature also played a crucial role in the arginase activity, the highest arginase production was observed at 37 °C, while both lower and higher temperatures led to reduced activity (Figure 5-D). Our study showed agreement with previous findings. For example, *Pseudomonas aeruginosa* showed the highest arginase activity at 37°C (Abd *et al*., 2025) and is not similar to *Bacillus thuringiensis* SK20.001, with an optimum at 40°C (Hernández *et al*., 2021).

Finally, our results show that the incubation time affected the enzyme levels, the enzyme production started at 24h, with the peak activity reached after 48 hr of cultivation. Prolonging the incubation beyond this point resulted in a slight decline in enzyme production (Figure 5-E). This temporal profile matches with observations for *Bacillus licheniformis* OF2 (Selim *et al*., 2024), but differs from *Pseudomonas aeruginosa* PV1, which reached maximum arginase production within 24 hrs (Nadaf & Vedamurthy, 2020). These differences show that the growth and metabolism of each strain affect how well its enzymes produce.

These differences display that the growth and metabolism of each strain affect its ability to produce enzymes. Arginase production that helps convert L-arginine into L-ornithine and urea (Malek *et al*., 2024؛ Abd *et al*., 2024) depends on the type of microorganism, where it was isolated from, and the geographical location of that source.

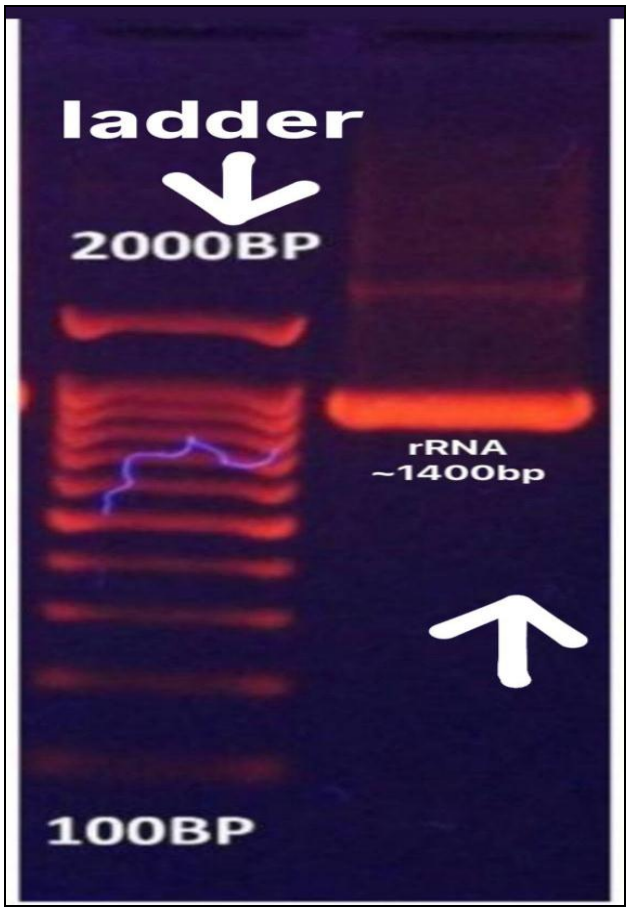

Fig. 1: The Gene Of 16S Rrna From An Unknown Isolate, Showed A Single Band Of Approximately 1400 Bp.

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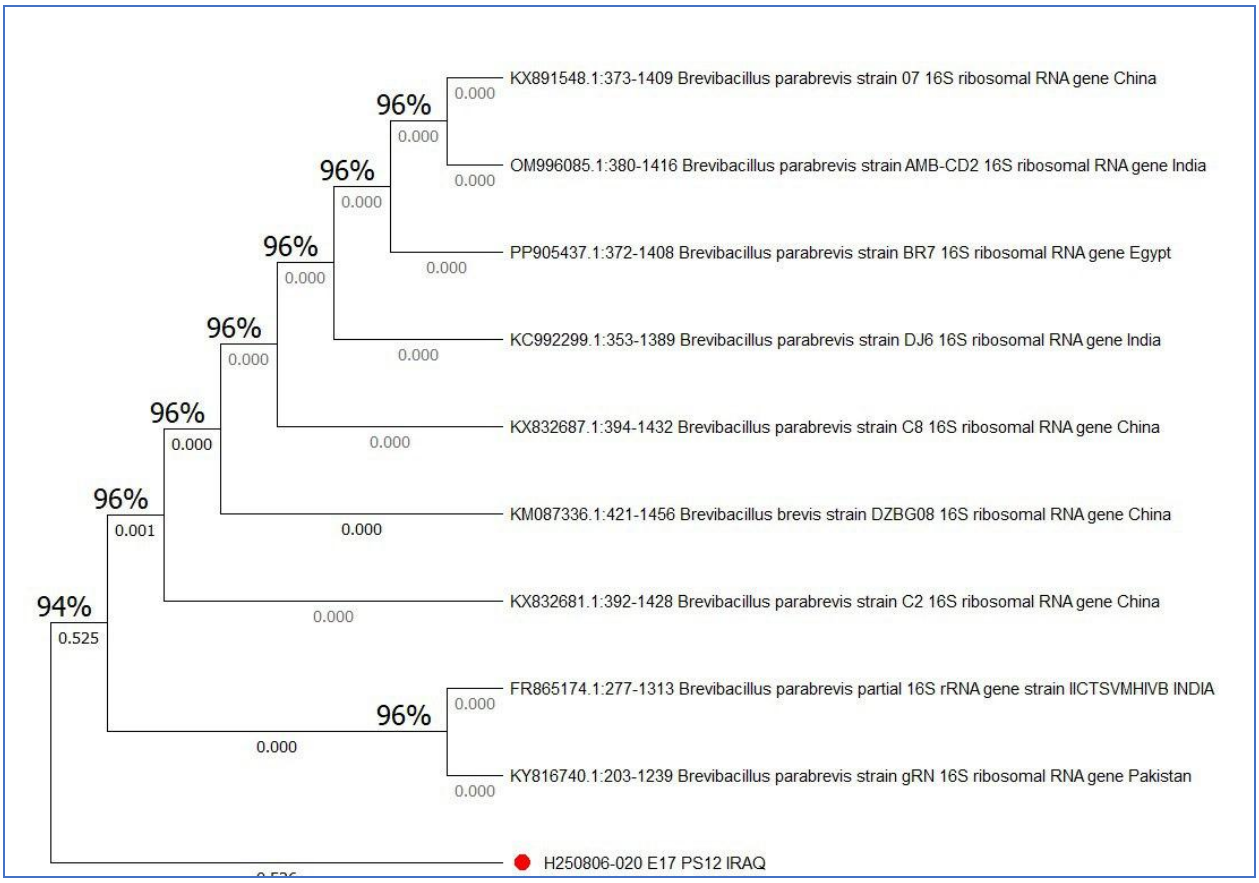

Fig. 2: Molecular phylogenic tree analysis

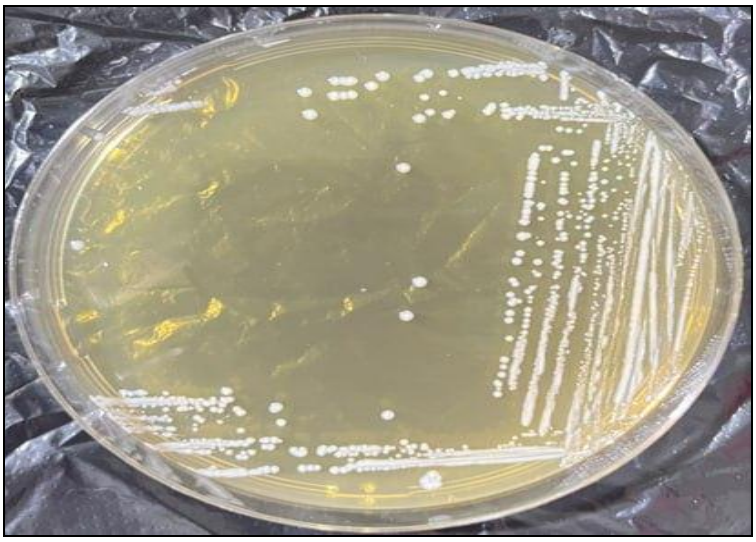

Fig. 3: *Brevibacillus parabrevis* on MRS medium

Fig. 4: Positive result of production arginase enzyme by *Brevibacillus parabrevis* PS12 on Modified Arginine Media.

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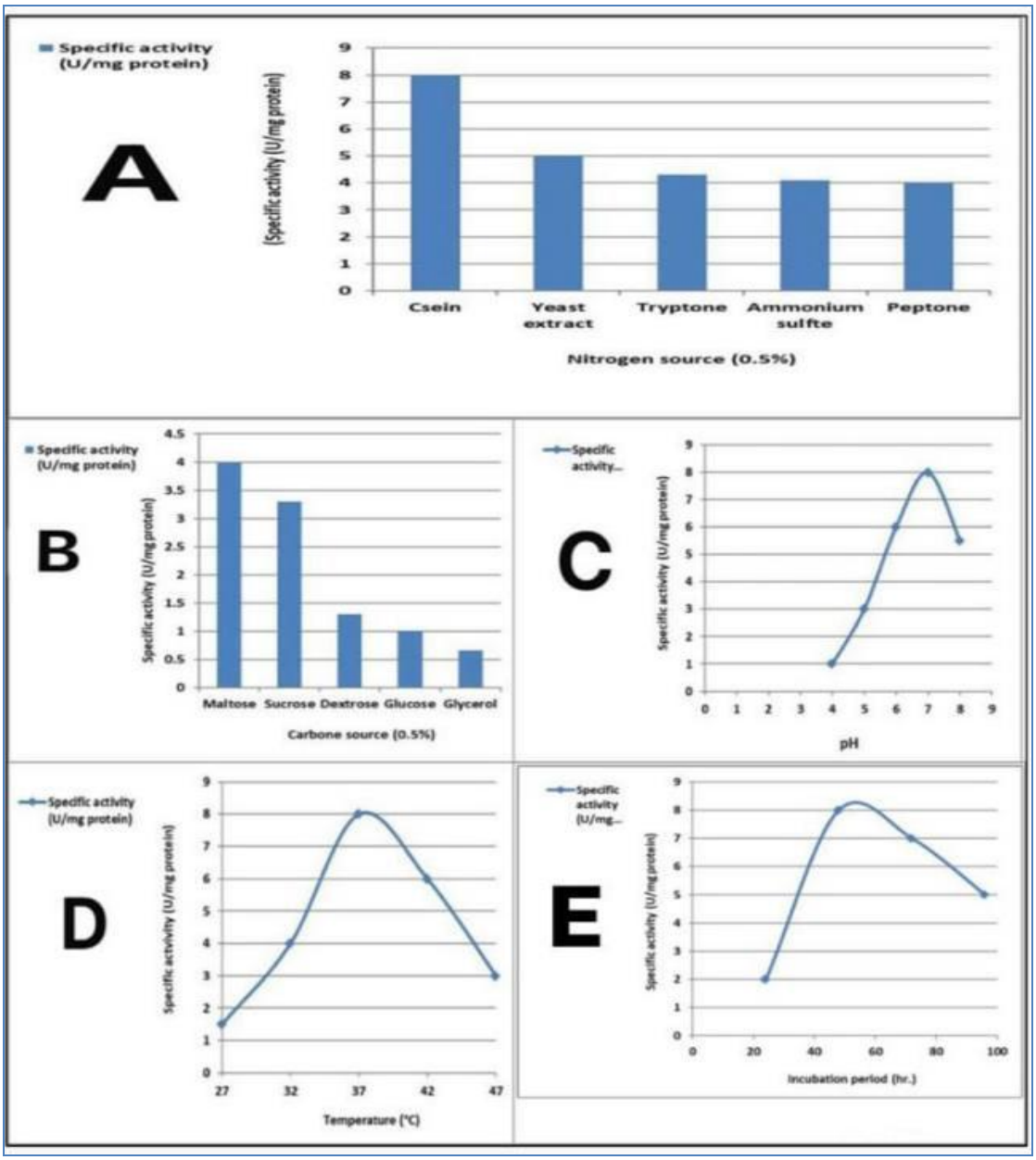

Fig. 5: Optimization of Factors Affecting on arginase specific activity**. (5-A).** Effect of nitrogen sources **(5-B).** Effect of carbon sources. **(5-C).** Effect of PH. **(5-D).** Effect of Temperature**. (5-E).** Effect of Incubation periods.

Conclusions

Brevibacillus parabrevis PS12 is a novel strain from Iraqi soil with unique genetic traits. It possesses high arginase activity and optimized arginase production highlights its biotechnological potential and provides a foundation for future studies on enzyme purification, characterization, and industrial applications.

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عزل سلالة جديدة *Brevibacillus parabrevis* PS12 من التربة وتحديد الظروف المثلى لإنتاج Arginase

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مستخلص البحث:
تُعرف أنواع بكتيريا *Brevibacillus* التي تعيش في التربة بتنوعها الأيضي، ويُكتشف أنها مصدرٌ للإنزيمات الطبيعية القيّمة للصناعة. يُحفّز إنزيم الأرجيناز عملية تحويل الأرجينين إلى إل-أورنيثين واليوريا، وهو الإنزيم الذي يلعب دورًا هامًا في أيض النيتروجين. يُعدّ إيجاد سلالات بكتيرية جديدة من مصادر بيئية قادرة على إنتاج إنزيم الأرجيناز أمرًا بالغ الأهمية لتوسيع نطاق الموارد الميكروبية للاستخدامات الصناعية والصيدلانية. من بين 20 عزلة، تم التعرف على عزلة واحدة وهي *Brevibacillus parabrevis* PS12 (97٪ تشابه SrRNA16، رقم الوصول GenBank (PX118439.1)، وهي الأولى التي تم الإبلاغ عنها من التربة العراقية. وصل نشاط ال Arginase إلى ذروته في المصدر الغذائي المالتوز كمصدر للكربون والكازين كمصدر للنيتروجين، كما أثرت العوامل الفيزيائية أيضًا على النشاط النوعي للإنزيم عند 7.0 درجة حموضة و37 درجة مئوية و48 ساعة من وقت الحضانة مع حاضنة هزازة 150 دورة في الدقيقة لضمان تجانس جميع مكونات الوسط والتهوية المستمرة ساهمت كل هذه الظروف في زيادة الكتلة الحيوية للبكتيريا مما أدى إلى ارتفاع النشاط النوعي لانزيم arginase.

كلمات مفتاحية: *Brevibacillus parabrevis* PS12، انزيم ارجنيز ، الظروف المثلى، وسط الأرجينين المعدني، تسلسل 16S Rrna
ملاحظة : البحث مستل من رسالة ماجستير.