

## Cytotoxic effect of purified amylase from *Bacillus licheniformis* on the MCF-7 cell line

Salam Muhil khafif<sup>(1)</sup>  
Mustansiriyah University,  
College of science,  
Baghdad, Iraq

Sawsan Hassan Authman<sup>(2)</sup>  
Mustansiriyah University,  
College of science,  
Baghdad, Iraq

Afrah Fahad Abdulkareem<sup>(3)</sup>  
Mustansiriyah University,  
College of science,  
Baghdad, Iraq

[salam2024@uomustansiriyah.edu.iq](mailto:salam2024@uomustansiriyah.edu.iq)

[dr.sawsanh@uomustansiriyah.edu.iq](mailto:dr.sawsanh@uomustansiriyah.edu.iq)

[aalfahad17@uomustansiriyah.edu.iq](mailto:aalfahad17@uomustansiriyah.edu.iq)

### Abstract

One of the main causes of cancer-related death for women globally is breast cancer. Interest in physiologically active enzymes has grown as a result of the hunt for less harmful and alternative medicinal treatments. The study aims to evaluate the potential cytotoxic effect of purified amylase on the proliferation of MCF-7 human breast cancer cells. The study included the extraction and purification of the amylase enzyme from *Bacillus licheniformis* from clinical and environmental sources, as 13 isolates were obtained from 145 samples collected from Al-Kindi Hospital from November 2024 to January 2025. Bacterial colonies were randomly streaked on freshly prepared starch agar plate's amylolytic strains and phenotypically assess *Bacillus licheniformis* ability to produce the amylase enzyme. The results showed that only 12 (95%) of *Bacillus licheniformis* could produce the amylase enzyme. Ammonium sulfate 80%, dialysis, and chromatography using Sephadex G150 and DEAE-cellulose columns were used to purify the amylase produced by *Bacillus licheniformis*. The purified amylase enzyme reduced the viability of MCF-7 breast cancer by 53.98% at a concentration of 150µg/ml.

**Keywords:** *Bacillus licheniformis*, Purification, Amylase, Anticancer.

### Introduction:

Predicting the function of enzymes is a particularly difficult task in bioinformatics. Enzymes are the most important molecules in our lives. They catalyze biological reactions for metabolism, organ structure, and cellular component maintenance (Yadav, S. K., & Tiwari, A. K. 2015). The four-part EC number is used by the International Union of Biochemistry and Molecular Biology enzyme classification system to identify an enzyme based on the

reaction it helps to carry out. Oxidoreductases (EC1), transferases (EC2), hydrolases (EC3), lyases (EC4), isomerases (EC5), and ligases (EC6) were the first six classified categories of enzymes. The "enzymes that cleave C-C, C-O, C-N, and other bonds by means other than by hydrolysis or oxidation" (lyases) are the most difficult to identify and categorize among them. Translocases (EC7) are a new class that was just discovered (McDonald, A. G., & Tipton, K. F. 2023). Amylase plays a crucial role in carbohydrate metabolism by catalyzing the cleavage of glycosidic linkages in polysaccharides, resulting in the formation of simple sugars units. The enzyme exists in three main forms  $\alpha$ ,  $\beta$ , and  $\gamma$  amylases-each acting at distinct positions within the carbohydrate chain (Akinfemiwa *et al.*, 2023). The most valuable sources of alpha amylase, an enzyme essential for the starch – processing industry, are various *Bacillus* species. Amylases derived from different Bacillus strains exhibit diverse properties, including variations in their optimal pH, temperature, and dependence on metal ions (Hu, Q., & Liu, J. 2021). The scientific name for  $\alpha$ -amylase is  $\alpha$ -1,4-glucan-4-glucanohydrolase, and its enzyme commission number is EC 3.2.1.1. Higher animals, plants, and microbes all secrete it. Calcium metal is utilized as a co-factor by a metalloenzyme, meaning that it is essential to its correct operation. Endo-amylase hydrolyzes the terminal glucose and  $\alpha$ -1,6-linkages and cleaves the  $\alpha$ -d-(1,4) glycosidic linkage. Starch is the substrate of  $\alpha$ -amylase (Farooq *et al.*, 2021). The second most fatal disease for women is breast cancer, which makes up 7–10% of all systemic malignant tumors and harms women physically and psychologically. At the time, endocrine therapy, chemotherapy, surgery, and radiation therapy were the available treatments for breast cancer. Patients with breast cancer have varying prognoses and responses to treatment because of the large number of subtypes of the disease and their clinical characteristics. Therefore, creating a new, effective, and universal treatment for breast cancer is essential. Poor therapeutic responses and prognoses persist despite advancements in therapy for various subtypes of breast cancer (Ramazi *et al.*, 2023).

Date from The World Health Organization indicate that breast cancer remains a major health concern, affecting more than 2.3 million women across the globe and is persistent in this population. In 2020, this disease claimed the lives of about 685,000 women (Sharmin *et al.*, 2021).

## Materials and method

### Collection of samples:

A total of 145 clinical and environmental samples were collected during November 2024 to January 2025 from Al-Kindi Hospital.

### Identification of *Bacillus licheniformis*:

#### Morphological Characteristics

All samples were cultivated on solid medium, such as nutritional agar and starch agar. Size, opacity, color, morphology, and margin were the criteria used to evaluate the colonies. Conducted these measurements over a 24-hour incubation period at 37°C (Shafique *et al.*, 2021).

#### Microscopical Examination

The bacterial isolates were examined under a light microscope at high power (100X) magnification while submerged in oil after being stained with gram stain to explore the general morphology of the cells (Rakaz *et al.*, 2021).

#### Biochemical Tests

To assess the enzymatic activity of the isolates, oxidase and catalase tests were conducted to confirm their ability to produce these enzymes (McDonald, A. G., & Tipton, K. F. 2023).

#### Identification using 16SrRNA

Kit's used in this Study include Master Mix and DNA Ladder (Promega / USA) to identification of *Bacillus licheniformis*.

Identification was confirmed by amplification of 16SrRNA gene by PCR using the following primers: F: 5'-GTGCCAGCAGCCGCGCTAA-3' and R: 5'-AGACCCGGGAACGTATTCAC-3' the PCR experiment was carried out using a thermocycler set to complete 40 cycles. The process started with an initial denaturation at 94°C for 5 minutes, followed by reported cycling that included denaturation at 94°C for 45 seconds, annealing at 50 °C for 45 seconds, and elongation at 72 °C for 7 minutes. (Maikalu *et al.*, 2023).

#### Phenotypic detection of amylase production

Amylase production was examined in purified isolates that grown on starch agar. Selected colonies were transferred onto freshly prepared starch agar plates and incubated at 50 °C for 48 hours. After the incubation period, the plates were treated with 1% iodine solution. After 5 to 10 minutes of being left undisturbed, the plates were decanted to remove the iodine solution. The diameter of the clear zone that formed surrounding the colonies was measured the isolates that had the widest clear zones surrounding them were chosen (Yassin *et al.*, 2021).

### Extraction of amylase

To inoculate the manufacturing medium, freshly made inoculum were utilized. A loop full of bacterial isolate was placed in 50 milliliters of inoculum medium that contained the following ingredients:  $\text{KH}_2\text{PO}_4$  0.05,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.015,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.25,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.05,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01, peptone 10, yeast extract 20, and starch 10 (g/L). The flask was kept at  $0^\circ\text{C}$  for 15 minutes at 7000 rpm in a rotary shaker incubator. Amylase was estimated using the obtained supernatant (Saini *et al.*, 2016).

### Purification of amylase

Several methods, including ammonium sulfate  $((\text{NH}_4)_2\text{SO}_4)$  precipitation, dialysis, DEAE-cellulose ion exchange chromatography, and gel filtering, were used to purify the crude amylase.

### Ammonium sulfate precipitation

A 0.1 M phosphate buffer with a pH of 6 was used to purify the crude enzyme from the supernatant fluid using ammonium sulfate. To do this, amounts of ammonium sulfate (80%) were utilized to precipitate the enzyme. The corresponding concentrations were combined with 200 milliliters of crude enzyme filtrate and maintained at  $4^\circ\text{C}$  for one to two hours while being constantly stirred. The precipitates were collected (Saini *et al.*, 2016).

### Gel filtration

The dialyzed enzyme fraction was further purified using gel filtration chromatography. It was applied to a Sephadex G- 150 column and eluted at a flow rate of 0.5 ml/min with Tris- HCl buffer (pH 7.4). Following the collection of total fractions (each 2 ml), the protein content was determined using a spectrophotometer set to 280. The fractions with the highest absorbance at 280 were collected, and their activity was assessed. For additional evaluation, the fractions exhibiting greater enzyme activity was gathered together (Saini *et al.*, 2016).

### Results and discussion:

#### Identification and isolation of *Bacillus licheniformis*:

*Bacillus licheniformis* has been collected from 145 clinical and environmental sites. *Bacillus licheniformis* was identified in 13 isolates using molecular identification using the 16SrRNA gene, biochemical assays, microscopic inspection, and culture. *Bacillus licheniformis* produced colonies that are remarkably variable in appearance and frequently seem to be mixed cultures. The colonies are moderately sized (2–4 mm) and irregular in shape, with a range of consistency from moist and/or mucoid (with undulate to fimbriae margins) to membranous with an underlying mucoid matrix to rough and crusty as they dry which agree with *Bacillus* isolates on nutrient agar

formed intermediate, slightly yellow colonies that were rough, flat, and had jagged edges (Malik *et al.*, 2022).



Figure (1) *Bacillus licheniformis* on nutrient agar

### Phenotypic detection of amylase production:

To examine for amylolytic strains, thirteen isolates of *Bacillus licheniformis* culture were streaked at random using a sterile wire loop on freshly prepared starch agar plates. For a full day, the plates were incubated at 37°C. Iodine solution staining was used to identify amylolytic microorganisms. The hydrolysis zone around the colonies contained amylase producers. Based on the results, 12 of the *Bacillus* isolates had a halo zone around their colony that produced amylase after iodine solution was introduced.

### Purification of amylase:

For the ammonium sulfate (80%) precipitation, the supernatant was collected. The specific activity and amylase activity of *Bacillus licheniformis*. Were 17.76 U/mg and 51.85 U/ml. The material was passed over a DEAE-cellulose column with a linear NaCl gradient (0.05–0.4 M). The maximum amylase activity was seen in the third protein peak, which reached 48.49 U/ml.

The amylase has been purified from a variety of sources using a DEAE-Cellulose Ion exchange column. It was used to purify the enzyme from *B. subtilis* ITBCCB148, yielding 47.4% with specific activity reach of 21.6 u/ml (Yandri *et al.*, 2010).

Further purification occurred by using Sephadex G150 for gel filtering. Once the enzyme fractions from DEAE-cellulose were combined and run through a gel filtration column, the fractionation yielded a single protein peak with an absorbance measurement at 280 nm. The amylase activity pooled and concentrated in the peak, reaching 47.85 U/ml.

**Table (1): Purification steps for amylase produced by *Bacillus licheniformis***

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield %
Ion exchange chromatography (DEAE-Cellulose)	15	48.49	1.5	32.33	727.35	5.59	7.16
Gel filtration	15	47.85	1.2	39.88	687.75	6.94	6.77

### Effect of amylase enzyme on MCF-7 cell line

Amylase's cytotoxicity was investigated in breast cancer cell lines (MCF-7) using the MTT assay. Amylase was used against of the breast cancer to ascertain the cytotoxicity profile. The concentration of the enzyme (150 µg/ml) had the greatest effect on cancer cells compared to other concentrations, reducing the viability of cancer cells to 33.20%. Amylase concentrations of (9.375, 18.75, 37.5, 75, and 150 µg/ml) had less impact on cancer cells, with 150 µg/ml reducing the viability of cancer cell lines to 53.98%.

**Table (2): MCF-7 Cell line (breast cell line cancer) 48 hrs Treatment**

MCF-7 Cell line ( breast cell line cancer ) 48 hrs treatment				
Concentration u/ml	Mean	Standard deviation	Survival fraction	Standard deviation of SF(SD)
Untreated	0.60	0.03	100	3.31
300	0.20	0.02	33.20	2.07
150	0.32	0.02	53.98	1.94
75	0.38	0.01	63.01	0.92
37.5	0.46	0.01	76.21	0.59
18.75	0.55	0.02	91.25	1.64
9.375	0.56	0.02	93.98	2.00
Blank				

Study by Abo-Kamer *et al.*, (2025) used the MTT assay to evaluate the cytotoxicity of amylase on Human hepatocellular carcinoma using HepG-2 cells revealing an IC<sub>50</sub> of 78.21 U/ml.

### Conclusion:

In the study, the amylase enzyme produced by *Bacillus licheniformis* was purified using various methods, including ammonium sulfate precipitation and gel filtration chromatography. These procedures contributed to obtaining a more purified enzyme, the anticancer activity of the amylase enzyme was assessed against the MCF-7 cell line. Results demonstrated that at a concentration of 150µg/ml, the enzyme significantly reduced the viability of cancer cells. These findings suggest that the enzyme possesses suitable properties for use in medical and industrial applications and provides a basis for future studies aimed at improving the purification process and enhancing enzyme efficiency. However, due to the limitations of the current study including the use of a single cell line and the absence of in vivo validation further comprehensive research is necessary to confirm these results and to explore the underlying mechanisms of action.

### References

1. Abo-Kamer, A. M., Abdelaziz, A. A., Elkotb, E. S., & Al-Madboly, L. A. (2025). Production and characterization of a promising microbial-derived lipase enzyme targeting BCL-2 gene expression in hepatocellular carcinoma. *Microbial Cell Factories*, 24(1), 58. 24:58 <https://doi.org/10.1186/s12934-025-02671-7>
2. Akinfemiwa, O., Zubair, M., & Muniraj, T. (2023). Amylase. *StatPearls*.
3. Farooq, M. A., Ali, S., Hassan, A., Tahir, H. M., Mumtaz, S., & Mumtaz, S. (2021). Biosynthesis and induction of amylase by *Bacillus subtilis* QM3. *Archives of Microbiology* (2021) 203:1281–1292. <https://doi.org/10.1007/s00203-020-02128-y>.
4. Hu, Q., & Liu, J. (2021). Production of  $\alpha$ -amylase by *Bacillus subtilis* QM3 and its enzymatic properties. *Open Access Library Journal*, 8(3), 1-8. DOI: 10.4236/oalib.1107291 Mar. 29, 2021
5. Malik, N. H. A., Simarani, K., & Aziz, M. A. (2022). Soybean as an alternative nutrient medium for *Bacillus subtilis* growth. *Malaysian Applied Biology*, 51(4), 67-74. <https://doi.org/10.55230/mabjournal.v51i4.12>
6. McDonald, A. G., & Tipton, K. F. (2023). Enzyme nomenclature and classification: the state of the art. *The FEBS journal*, 290(9), 2214-2231. doi:10.1111/febs.16274.
7. Maikal R B, Igere B E, Odjadjare E E. Enterobacter species distribution, emerging virulence and multiple antibiotic resistance dynamics in effluents: A countrified spread-hub and implications of abattior release. *Total Environment Research Themes*. 2023; 8 100074. <https://doi.org/10.1016/j.totert.2023.100074>
8. Rakaz, M. A., Hussien, M. O., & Ibrahim, H. M. (2021). Isolation, Extraction, Purification, and Molecular Characterization for Thermostable  $\alpha$ -Amylase from Locally Isolated *Bacillus* Species in Sudan. *Biochemistry research international*, 2021(1), 6670380. Hindawi Biochemistry Research International Volume 2021, Article ID 6670380, 8 pages <https://doi.org/10.1155/2021/6670380>
9. Ramazi, S., Salimian, M., Allahverdi, A., Kianamiri, S., & Abdolmaleki, P. (2023). Synergistic cytotoxic effects of an extremely low-frequency electromagnetic field with doxorubicin on MCF-7 cell line. *Scientific Reports*, 13(1), 8844.
10. Saini, H., Saini, R., Dahiya, A., & Mehta, S. (2016). Extraction, partial purification and characterization of amylase from apple (*Malus pumila*). *International Journal of Food and Nutritional Science*, 5(3).



11. Shafique, T., Shafique, J., Zahid, S., Kazi, M., Alnemer, O., & Ahmad, A. (2021). Screening, selection and development of Bacillus subtilis apr-IBL04 for hyper production of macromolecule alkaline protease. Saudi journal of biological sciences, 28(2), 1494-1501. <https://doi.org/10.1016/j.sjbs.2020.11.079>
12. Sharmin, S., Rahaman, M. M., Martorell, M., Sastre-Serra, J., Sharifi-Rad, J., Butnariu, M., ... & Islam, M. T. (2021). Cytotoxicity of synthetic derivatives against breast cancer and multi-drug resistant breast cancer cell lines: A literature-based perspective study. *Cancer Cell International*, 21(1), 612.
13. Yadav, S. K., & Tiwari, A. K. (2015). Classification of enzymes using machine learning based approaches: a review. *Machine Learning and Applications*, 2(3/4), 30-49. DOI : 10.5121/mlaj.2015.2404
14. Yandri, T. S., & Hadi, S. U. T. O. P. O. (2010). Immobilization of  $\alpha$ -amylase from locale bacteria isolate Bacillus subtilis ITBCCB148 with diethylaminoethyl cellulose (DEAE-Cellulose). *Material Science Research India*, 7(1), 123-128.
15. Yassin, S. N., Jiru, T. M., & Indracanti, M. (2021). Screening and Characterization of Thermostable Amylase-Producing Bacteria Isolated from Soil Samples of Afdera, Afar Region, and Molecular Detection of Amylase-Coding Gene. *International journal of microbiology*, 2021(1), 5592885. Hindawi International Journal of Microbiology Volume 2021, Article ID 5592885, 14 pages <https://doi.org/10.1155/2021/5592885>

**التأثير السمي الخلوي لأنزيم الاميليز المنقى من بكتيريا *Bacillus licheniformis* على خلايا MCF-7**

قسم علوم الحياة ، كلية العلوم ، الجامعة المستنصرية، العراق  
قسم علوم الحياة ، كلية العلوم ، الجامعة المستنصرية، العراق  
قسم علوم الحياة ، كلية العلوم ، الجامعة المستنصرية، العراق

سلام محيل خفيف  
د. سوسن حسن عثمان  
د. افراح فهد عبد الكريم

[salam2024@uomustansiriyah.edu.iq](mailto:salam2024@uomustansiriyah.edu.iq)  
[dr.sawsanh@uomustansiriyah.edu.iq](mailto:dr.sawsanh@uomustansiriyah.edu.iq)  
[aalfahad17@uomustansiriyah.edu.iq](mailto:aalfahad17@uomustansiriyah.edu.iq)

**مستخلص البحث:**

يعد سرطان الثدي أحد الاسباب الرئيسية للوفيات المرتبطة بالسرطان لدى النساء على مستوى العالم وقد ازداد الاهتمام بالانزيمات النشطة فسيولوجيا نتيجتا للبحث عن علاجات طبية بديلة وأقل ضررا. تهدف الدراسة الى تقييم التأثير السام المحتمل للأميليز المنقى على تكاثر خلايا سرطان الثدي البشرية . شملت الدراسة استخلاص وتنقية انزيم الاميليز من بكتيريا MCF-7 من مصادر سريرية وبيئية ، حيث تم الحصول على 13 عزلة بكتيرية *Bacillus licheniformis* من 145 عينة جمعت من مستشفى الكندي خلال الفترة من تشرين الاول 2024 الى كانون الاول 2025. وضعت المستعمرات البكتيرية عشوائيا في اطباق اجار النشا المحضرة حديثا وقيمت قدرة على انتاج انزيم الاميليز. اظهرت النتائج ان 12(95%) فقط من *Bacillus licheniformis* قادرة على انتاج انزيم الاميليز. استخدمت كبريتات الامونيوم 80%، *Bacillus licheniformis* DEAE-Cellulose, Sephadex- G150 وتقنية الديلزة والكروماتوغرافيا باستخدام اعمدة للبقاء على قيد الحياة MCF-7 لتنقية انزيم الاميليز. قلل انزيم الاميليز من قابلية سرطان الثدي بنسبة 53.98% عند تركيز 150 مايكروغرام/مل. **الكلمات المفتاحية:** العسوية الحزازية ، التنقية، الاميليز، مضاد للسرطان .