PRODUCTION OF A-AMYLASE FROM *GEOBACILLUS STEAROTHERMOPHILUS* AND ITS APPLICATION IN FOOD WASTE BIODEGRADATION

Dedan M.amin Abdalhakim **Khanzad Khudhur jarjees**

*Department of Food Technology; College of Agriculture Engineering Sciences Salahaddin University Erbil Iraq.

Abstract:

Agriculture and industrial waste are one of the most components that accumulate in the environment according to the activity of countries. Therefore, these wastes can be used as substrates to produce useful products like amylase enzyme as a friendly solution for this problem. The present research aimed to isolate a total of 100 bacterial isolates (50 from each soil type location) were primarily selected for the isolation of bacteria. Among them, the percentage of *G. stearothermophilus* bacteria was higher in Kirkuk soil than in Erbil soil, Bacterial growth was observed on cultured nutrient agar (14 that % 28) of positive bacteria were in Erbil government and (21 that % 42) of positive bacteria were in Kirkuk government. The ability of these isolates was tested for starch degradation using a solid starch medium at 55 °C for 2 days. Results indicated that only thirty-five isolates were selected for their capability to produce amylase enzyme according to the diameter (mm) of clearing zones. Secondary screening of thirty-five isolates was achieved in a submerged liquid production medium with 1% soluble starch at pH (7) and 55° C for 2. All isolated bacteria were identified according to morphological, and biochemical characteristics and by VITEK® 2 Compact BCL kit. Four agriculture wastes (wheat bran, broken rice, banana peel and potato peel) were used as a substrate for amylase production, the most active isolates AB1, AB43 and AB97 were inoculated in a submerged liquid production medium with 1% of each waste at 55˚C for 2 days, then quantitative of amylase was determined, which shows that potato peel had a greater result among all other substrate and all food waste had a greater result than positive control which was Nutrient broth with adding %1 soluble starch.

Keywords: Agriculture wastes, *Geobacillus stearothermophilus,* α-amylase, PCR, *Gt-amy* gene.

انتاج أنزيم الفا امايليز من قبل بكتريا ***Geobacillus stearothermophilus***وتطبيقه في التحلل البيولوجي لفضلات الطعام.

ديدەن محمدامين عبدالحكيم آ.م.د خانزاد خضر جرجيس

الباحث استاذ المساعد

قسم تكنولوجيا الغذاء/اقليم كردستان/ كلية علوم الهندسة الزراعية/ جامعة صلاح الدين.

الخلاصة:

تعتبر النفايات الزراعية والصناعية من أكثر المكونات التي تتراكم في البيئة حسب نشاط الدول. لذلك، يمكن استخدام هذه النفايات كركائز لإنتاج منتجات مفيدة مثل إنزيم الأميليز كحل مناسب لهذه المشكلة. الأميليز هو إنزيم مهم في العديد من التطبيقات لتحويل الكربوهيدرات المعقدة إلى سكريات بسيطة. يهدف البحث الحالي إلى عزل العازلات البكتيرية المحبة للحرارة من عينات التربة، ثم اختبارها لإنتاج الأميليز ودراسة قدرتها على إنتاج الأميليز باستخدام أربع مخلفات زراعية. تم فحص مئات العازلات البكتيرية المحلية من *G. stearothermophilus* المحبة للحرارة المعزولة من مواقع مختلفة من التربة كردستان العراق في) أربيل وكركوك (لقدرتها على إنتاج α-amylase في الأوساط الصلبة والسائلة ونفايات الطعام المزروعة. تم اختيار العزلة *G. stearothermophilus* بناءً على إنتاجها من الإنزيم بين العازلات الأخرى واستخدمت في العمل الحالي لدراسة إنتاج وتنقية وتوصيف الإنزيم والكشف عن جين α-amylase. تم اختيار مجموعه 100 عزلة بكتيرية (50 من كل موقع من أنواع التربة) بشكل أساسي لقدرتها على التحلل النشواني. من بينها، كانت نسبة بكتريا *G. stearothermophilus* أعلى في تربة كركوك منها في تربة أربيل، ولوحظ نمو بكتيري على أجار المغذيات المستزرعة (14٪ 28) من البكتيريا الموجبة في مدينه أربيل و (21٪ 42) من البكتيريا الموجبة كانوا في مدينه كركوك. تم اختبار قدرة هذه العازلات على تحلل النشا باستخدام وسط نشا صلب عند 55 درجة مئوية لمدة يومين. أشارت النتائج إلى أنه تم اختيار 35 عزلة فقط لقدرتها على إنتاج إنزيم الأميليز وفقًا لقطر (مم) من مناطق المقاصة. تم إجراء غربلة ثانوية لـ 35 عزلة في وسط إنتاج سائل مغمور بنشا قابل للذوبان بنسبة 1٪ عند درجة حموضة (7) و55 درجة مئوية لمدة يومين في حاضنة بسرعة 150 دورة في الدقيقة. تم تحديد جميع البكتيريا المعزولة وفقًا للخصائص المورفولوجية والكيميائية الحيوية وبواسطة مجموعة VITEK® 2 Compact BCL KIT والتي تستخدم فقط مع Bacillus spp. تم استخدام أربع نفايات زراعية (نخالة القمح والأرز المكسور وقشر الموز وقشر البطاطس) كركيزة لإنتاج الأميليز، وتم تلقيح العازلات الأكثر نشاطًا 1AB و43AB و97AB في وسط إنتاج سائل مغمور بنسبة 1 ٪ من كل نفايات عند 55 درجة مئوية و150 دورة في الدقيقة لمدة يومين، ثم تم تحديد كمية الأميليز ومقارنتها بالنشا الذائب.

1. Introduction

In spite of progress in biotechnology and enzymology, the enzymes have been industrialized in recent years for the mounting up product development in the various area (13). α- Amylases are enzymes that catalyse the hydrolysis of internal α-1, 4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose and maltotriose units (25). Amylases can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (29). It can be used in the field related to biotechnology such as: removing environmental pollutants, conversion of starch to the desired substrate by many microorganisms, infiltration of waste containing starch and production of

biochemical material with helping starch substrate (22). Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (23). Enzymes production from thermophilic hosts has several advantages compared with mesophilic hosts, including reduced cooling costs, lower contamination risk, increased substrate and product solubility, and temperature optima of these bacteria matching those of enzymes used for simultaneous saccharification and fermentation (21).

Glazer and Nikaido, reported that agricultural wastes, such as wheat straw, corn cobs, oat hulls, and sugarcane wastes represent a large number of organic wastes (8). Organic wastes can be considered microbial food sources or substrates. (Maier *and* Sidkey) used different fermented enviro-agro-industrial wastes as very cheap and available substrates for obtaining microbial α-amylases (16;26). Most species are modest bacteria able to develop without growth factors or vitamins and to utilize alkanes as carbon and energy sources (28). Growth at high temperatures makes *Geobacillus* species promising agents in biotechnological processes. The enzymes from thermophilic microorganisms are extremely in high demand for industries such as biocatalysts, biotransformation and biodegradation. Thermostable enzymes are typically hard to denature at elevated temperatures (>50 C) while increasing the temperature will disrupt the shape of the active site, which will reduce the activity of the enzyme (33).

The aims of this study are:
To isolate thermophilic *G. stearothermophilus* from the soil in different sights of Erbil and Kirkuk district area. Also, to determine the α-amylase production by this bacterium and Applicate these bacteria in food waste such as wheat and broken rice and potato and banana pill. Molecular identification of *Gt-amy* gene by PCR.

2.2 MATERIALS AND METHODS:

2.2.1 Sample collection and culture initiation

The 100 soil samples were collected from different district areas in Kurdistan-Iraq (Erbil and Kirkuk governments). The bacterial colonies were isolated through the serial dilution method. Briefly, one gram of soil was suspended in 9 mL of distilled water and was further diluted five times. The samples were then spread on Nutrient agar (NA) plates and incubated at 55° C for 24–48 hrs. The plates were examined after every 12 hrs. to select and inoculate the bacterial colonies on new NA plates to get pure colonies. The bacterial colonies were further preserved as liquid cultures in Nutrient Broth media at 4 C and as glycerol stocks at 80° C.

2.2.2 Morphological and biochemical tests:

The morphology of the colonies or colonial characteristics of the isolated bacterial strains such as size, shape, margin, elevation, opacity, and colour were observed macroscopically. The bacterial colonies were streaked on NA and were observed for different morphological characteristics after 24 hr. of incubation. The gram staining was performed using fresh cultures after 18–24 hrs. of growth following the standard procedures (4). Bacterial colonies were identified using morphological characteristics, microscopic appearances and a set of biochemical tests (31).

2.2.3 VITEK 2 compact system and BCL card

A VITEK2 instrument was used for the initial testing, and subsequent retests used a VITEK2 Compact instrument. Bacterial suspensions were prepared in 2.5 ml of sterile saline and adjusted to a McFarland standard of 1.8–2.2 using the VITEK 2 DensiChek (bioMe´rieux). BCL cards were filled automatically in the VITEK vacuum chamber, sealed, incubated at 55° C and read automatically every 15 min for 14 h. Data were analyzed automatically using the VITEK 2 database version 08.01, original data were compared with it (10).

2.2.4 Screening of amylase-producing ability

The ability of bacteria to produce amylase was observed based on their ability to degrade starch. Each bacterial isolate was inoculated on Nutrient agar media containing starch for 48 hours at the temperature of their original habitat and then tested by dripping iodine solution around the bacterial colony. The ability to produce amylase was demonstrated by the formation of clear zones around bacterial colonies. The isolate producing the largest clear zone ratio was chosen for the next subsequent step. Statistical analysis of the clear zone ratio used SPSS Duncan's multiple range tests. The diameters of the halo zone were measured for selected single colonies and the ratio between the diameter of the halo zone to colony diameter for each isolate was determined (17).

Starch hydrolysis ratio (SHR) = Zone diameter (mm) ÷ Colony diameter (mm).

The isolate producing the largest clear zone ratio was chosen for the next subsequent step. Statistical analysis of the clear zone ratio used SPSS Duncan's multiple range test with a level of 5

2.2.5 Enzyme activity assay

The digestive activity of α-amylases is measured via several colorimetric methods, including the dinitro salicylic acid method (DNS), and the Iodine method. DNS is an alkaline reagent that attaches to the reducing sugars and then color changes can be detected by UV absorbance at 540 nm (9). (Figure 1).

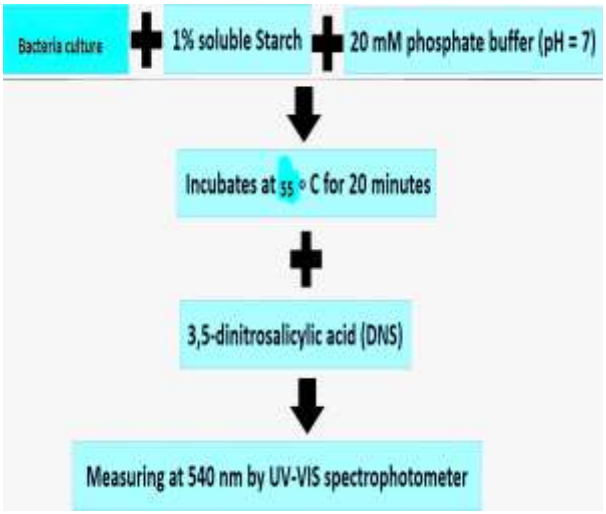

Figure 1. Assaying of α-amylase activity by DNS method.

2.2.6 PCR amplification of *Gt-amy* gene

A set of oligonucleotide primers (*Gt-amy* F:5'-GCCGATGTCGTGTTCGACCAT-3', and *Gt-amy* R: 5'-TGACGGCATCAAGCCGGA-3') were designed based on the conserved nucleotide sequences of thermostable α-

amylases from *Bacillus* and *Geobacillus* spp. For amplifying partial conserved domain sequence.

PCR amplification was carried out using primers designed from the end regions of the α-amylases for obtaining the complete (1650 bp). Approximately 50–100 ng of genomic DNA was used as a template for the amplification of the *Gt-amy* in a Thermocycler (Bio-Rad, USA) in a 50 µL reaction mixture (initial denaturation at 95° C for 5 min followed by 30 cycles of denaturation at 94° C for 50 s, annealing at 60° C for 40 s, extension at 72° C for 2 min and a final extension at 72° C for 5 min) (19).

2.2.7 Amylase production by agricultural wastes:

The three selected isolates from secondary screening were used to produce amylase enzyme using four agricultural wastes as a carbon source in a liquid production medium. 1% from each waste was added to 25 ml of liquid production medium without soluble starch and inoculated with 1 ml from overnight grown cultures of three isolates which have approximately $1 \pm 0.2 \times 10^7$ cell/ml and incubated at 50° C for 2 days in a shaker incubator at 150 rpm. After the incubation period, the bacterial cultures were harvested by centrifugation at 6000 rpm for 20 min. and the supernatants (culture filtrate) were used to determine the enzymatic activity by measuring the absorbance at 600 nm (12).

3. RESULTS AND DISCUSSION:

3.1 Isolation and morphological:

The incubation of serially diluted soil sample in the Nutrient agar plates for 48 hrs. at 55 C resulted in the isolation of bacterial colonies. The pure cultures of these isolates were further debated for macroscopic observations. Morphological traits such as colony colour, shape, margins, elevation, and opacity were carefully observed to characterize the bacteria. The different bacterial isolates were named AB. Between those isolates, the strain was chosen for further work. Its colonies were round, whitish, with smooth margins, and a diameter of 3–4 mm. It forms gram-positive, spore-forming as shown in Figure (4.1)

Figure (4.1) Bacterial Growth 48h

3.2 Identification of Bacterial Isolates

3.2.1 The morphological features:

The morphological features of the colonies were flat, opaque, rough surfaces and they had the circular irregular edge. All isolates were Gram-positive, rod-shaped; spore-forming bacteria after the microscopical examination was done (Fig 4.2) (2).

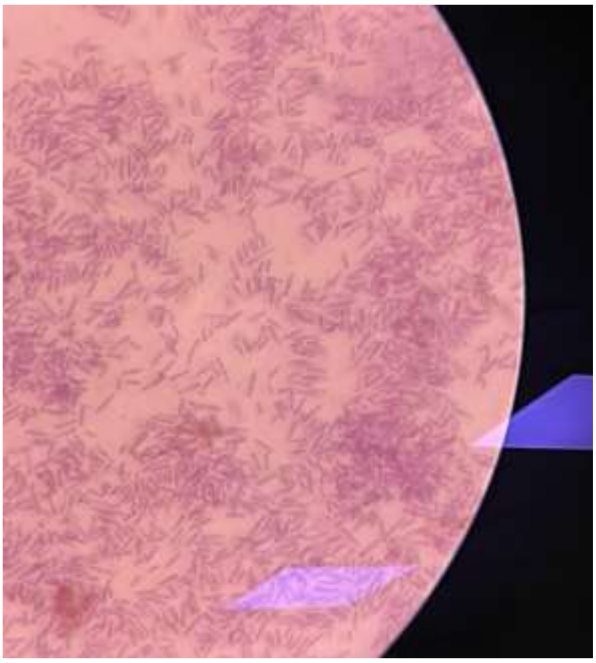

Figure (4.2): *G. sterothermophilus* under the microscope

3.2.2 chemical identification:

3.2.2.1 Catalase activity: was determined by the formation of oxygen bubbles with a 3% hydrogen peroxide solution.

3.2.2.2 Oxidase activity: was determined by the oxidation of TMPD (tetramethyl-phenylenediamine dihydrochloride) provided in form of discs.

3.2.2.3 starch hydrolysis was tested by flooding Gram's iodine on the colonies of the strains grown on the starch agar containing 1% starch and observing the presence or absence of clearing around the colony. Show in the table (4.1) for the Erbil government and (4.2) for the Kirkuk government (5).

3.2.2.4 Carbohydrates fermentation (Parry et al., 1983)

Bacteria were grown on a Nutrient broth medium containing the sugar to be tested and incubated at 55C^{o} for 3-5 days, the turn of the reagent's colour into yellow indicated utilization of the sugar and the forming of an acid.

Table (4-1): Identification of isolated *G. stearothermophlilus* in Erbil Government

Isolate No.	Gram stain	Chemical tests			
		1	2	3	4
AB 1	+	+	+	+	+
AB2	+	+	-	+	+
AB3	+	+	+	+	+
AB4	+	+	+	-	-
AB5	-	+	+	-	-
AB6	-	+	+	-	-
AB7	-	+	+	-	-
AB8	+	+	-	+	-
AB9	+	+	+	+	+
AB10	-	+	+	-	-
AB11	-	+	+	-	-
AB12	-	+	+	-	-
AB13	+	+	+	-	-
AB14	+	+	-	+	-
AB15	-	+	+	-	-
AB16	-	+	+	-	-
AB17	+	+	+	+	+
AB18	+	-	-	+	-
AB19	+	+	+	+	+
AB20	-	-	-	-	-
AB21	+	+	+	+	+
AB22	+	+	+	+	+
AB23	-	-	-	-	-
AB24	+	-	-	-	-
AB25	+	+	+	+	+
AB26	-	-	+	+	-
AB27	+	+	+	+	+
AB28	+	+	+	+	+
AB29	+	+	-	+	-
AB30	-	-	+	+	-
AB31	+	+	+	-	+
AB32	+	+	+	+	+
AB33	-	-	+	-	-
AB34	+	-	-	+	+
AB35	+	+	+	-	-
AB36	+	-	+	+	-
AB37	-	+	+	-	-
AB38	+	+	+	-	+
AB39	+	+	+	+	+
AB40	-	+	+	-	-
AB41	+	-	+	+	-
AB42	-	+	+	-	-
AB43	+	+	+	+	+
AB44	-	+	+	-	+
AB45	-	+	+	-	-
AB46	+	+	+	-	+
AB47	+	+	-	+	-
AB48	-	+	+	-	-
AB49	+	+	+	-	-
AB50	+	+	+	+	+

1; Catalase test, 2; Oxidase test, 3; Starch hydrolase test, 4; Carbohydrates fermentation.

(4.2) Identification of isolated G. *stearothermophlilus* in Kirkuk Government

Isolate No.	Gram stain	Chemical tests			
		1	2	3	4
AB51	+	+	+	+	+
AB52	+	+	+	+	+
AB53	+	+	+	+	+
AB54	+	+	+	-	-
AB55	-	+	+	-	-
AB56	-	+	+	-	-
AB57	-	+	+	-	-
AB58	+	+	+	-	-
AB59	+	+	+	+	+
AB60	-	+	+	-	+
AB61	-	+	+	-	-
AB62	-	+	+	-	-
AB63	+	+	+	+	+
AB64	+	+	+	+	+
AB65	+	+	+	+	+
AB66	-	+	+	-	+
AB67	+	+	+	+	+
AB68	+	-	-	+	-
AB69	+	+	+	+	+
AB70	-	-	-	-	-
AB71	+	+	+	+	+
AB72	+	+	-	+	-
AB73	-	-	-	-	-
AB74	+	-	-	-	-
AB75	+	+	+	+	+
AB76	-	-	+	+	-

AB77	+	+	+	+	+
AB78	-	-	+	-	-
AB79	+	+	+	+	+
AB80	-	-	+	+	-
AB81	+	+	+	+	+
AB82	+	+	+	+	+
AB83	-	-	+	-	-
AB84	+	-	-	+	-
AB85	+	+	+	+	+
AB86	-	+	+	-	-
AB87	-	+	+	-	+
AB88	+	+	+	+	+
AB89	+	+	+	+	+
AB90	-	+	+	-	-
AB91	-	+	+	-	-
AB92	-	+	+	-	-
AB93	+	+	+	+	+
AB94	-	+	+	-	+
AB95	-	+	+	-	-
AB96	-	+	+	-	-
AB97	+	+	+	+	+
AB98	-	+	+	-	+
AB99	+	+	+	-	-
AB100	+	+	+	+	+

1; Catalase test, 2; Oxidase test, 3; Starch hydrolase test, 4; Carbohydrates fermentation.

3.2.3 VITEK® 2 Compact

For further investigation and the identification of *G. stearothermophilus* VITEK® 2 compact system was applied. Figure (4.3) shows the VITEK® 2 card after analyzing the samples. After the required time for the identification of the isolates, the results were registered in the VITEK® 2 compact system program and printed. The probability of the results was high in the range (99%) with excellent identification of *G. stearothermophilus* species.

Figure (4.3): Sample unit of Vitek®2 BCL Kit after use

(Halket et al., 2010) used the VITEK® 2 system (BCL) card for bacillus species which showed %100 identification for *G. stearothermophilus* (10). Overall, in this study, all selected isolate has been identified in an excellent range. which has been done 46 different chemical tests on bacteria in a short time if we do all these tests by ourselves will need more time and more economical needs.so **VITEK® 2 Compact system** is one of the

most ideal ways to the identification of Bactria and it also prevents contamination during the processing of tests. All specific tests that the BCL kit will do it shows in the table (4.3).

Table (4.3) Shows test substrates on BCL Card.

Well	Test	Mnemonic	result
1	BETA−XYLOSIDASE	BXYL	-
2	L−Lycine−ARYLAMIDASE	LycA	-
3	L−Acpartate ARYLAMIDASE	AcpA	+
4	Leucine−ARYLAMIDASE	LeuA	+
5	Phenyl alanine ARYLAMIDASE	PheA	+
6	L−Proline ARYLAMIDASE	ProA	-
7	BETA−GALACTOSIDASE	BGAL	-
8	L−PyrroLydonyL−ARYLAMIDASE	PyrA	+
9	ALPHA−GALACTOSIDASE	AGAL	+
10	ALanine ARYLAMIDASE	ALaA	+
11	Tyrocine ARYLAMIDASE	TyrA	+
12	BETA−N−ACETYL−GLUCOSAMINIDASE	BNAG	-
13	ALa−Phe−Pro ARYLAMIDASE	APPA	+
14	CYCLODEXTRIN	CDEX	-
15	D−GALACTOSE	dGAL	+
16	GLYCOGEN	GLYG	-
17	myo−INOSITOL	INO	-
18	METHYL−A−D−GLUCOPYRANOSIDE acidification	MdG	+
19	ELLMAN	ELLM	+
20	METHYL−D−XYLOSIDE	MdX	+
21	ALPHA−MANNOSIDASE	AMAN	-
22	MALTOTRIOSE	MTE	+
23	GLycine ARYLAMIDASE	GLyA	-
24	D−MANNITOL	dMAN	-
25	D−MANNOSE	dMNE	+
26	D−MELEZITOSE	dMLZ	+
27	N−ACETYL−D−GLUCOSAMINE	NAG	+
28	PALATINOSE	PLE	+
25	L−RHAMNOSE	IRHA	-
30	BETA−GLUCOSIDASE	BGLU	+
31	BETA−MANNOSIDASE	BMAN	-
32	PHOSPHORYL CHOLINE	PHC	-
33	PYRUVATE	PVATE	+
34	ALPHA−GLUCOSIDASE	AGLU	+
35	D−TAGATOSE	dTAG	-
36	D−TREHALOSE	dTRE	-
37	INULIN	INU	-
38	D−GLUCOSE	dGLU	+
39	D−RIBOSE	dRIB	-
40	PUTRESCINE accimiLation	PSCNa	-
41	GROWTH IN 6.5% NaCL	NaCL 6.5%	-
42	KANAMYCIN RESISTANCE	KAN	+
43	OLEANDOMYCIN RESISTANCE	OLD	-
44	ESCULIN hydroLycic	ESC	+
45	TETRAZOLIUM RED	TTZ	-
46	POLYMIXIN_B RESISTANCE	POLYB_R	-

3.3 α-amylase Production assay:

3.3.1 Primary screening for amylase:

After identification of *G. stearothermophilus* all 35 isolate bacteria have been chosen for screening of amylase activity**,** isolates were α-amylase producing according to the clear zone around the growing colony on the 1.5% starch for 24 hours at 55^{o} C (15). Eight isolates were recognized by the higher production of the α-amylase on the solid media supplemented with 1.5% soluble starch as shown in the table (4.4

Table (4.4): Screening of *G. stearothermophilus* isolates for their ability to produce α-amylase.

Isolate No.	G diameter (mm)	H (clear zone) diameter (mm)	H/ G ratio	Isolate No.	G diameter (mm)	H (clear zone) diameter (mm)	H/ G ratio
AB 1	2	12	6	*AB63*	3	4	1.33
AB3	2	14	7	*AB64*	2	14	7
AB9	4	13	3.25	*AB65*	5	16	3.2
AB17	4	12	3	*AB67*	3	14	4.66
AB19	4	16	4	*AB69*	5	16	3.2
AB21	3	14	4.66	*AB71*	3	14	4.66
AB22	3	14	4.66	*AB75*	3	12	4
AB25	4	15	3.75	*AB77*	4	13	3.25
AB27	3	14	4.66	*AB79*	4	15	3.75
AB28	2	13	6.5	*AB81*	3	14	4.66
AB32	2	15	7.5	*AB82*	4	16	4
AB39	4	15	3.75	*AB85*	3	14	4.66
AB43	3	18	6	*AB88*	3	14	4.66
AB50	3	16	5.33	*AB89*	4	15	3.75
AB51	4	13	3.25	*AB93*	3	12	4
AB52	3	12	4	*AB97*	2	16	8
AB53	4	15	3.75	*AB100*	4	16	4
AB59	3	20	6.66				

H: halo, clear zone

G: Growth diameter

3.3.2 Secondary screening:

The ability of *G. stearothermophilus* isolates to produce α-amylase enzyme was tested by using a fermentation medium. It might be due to the fact that this fermentation medium provided an adequate amount of nutrients necessary for the production of α-amylase as Sema and Barıs¸ Enez have done before. medium supplemented with 1.5 % starch to determine the optimal temperature for the α-amylase activity assay. The results, shown in table (4.5) for the specific activity of α-amylase, produced by the eight selected isolates **AB1, AB3, AB28, AB32, AB43, AB59, AB64, and AB97**.That three isolates were the highest among them, which were AB3, AB43, and AB97 had measured 126.60, 130.23 and 169.33 unit\ml respectively in the crude

extract, produced by this isolate and it was after incubation at 55 ºC for 24 hrs. (Sarabjeet and Sema and Barıs) have also reported the production of α-amylase by same species (27; 3). Hence, these isolates were selected for further experiments to produce amylase enzymes by using agricultural wastes. *G. stearothermophilus* can produce large amounts of extracellular enzymes such as amylases and proteases, the α-amylase is highly thermostable and is desirable for industrial applications (20; 6).

Table (4.5) Secondary screening of α-amylase produces by *G. stearothermophilus* in Nutrient broth by adding %1 soluble starch.

No.	Isolates	Conc. (**µg/ml)**	Enzyme activity (Unit\ ml)
1	AB1	21665.8263	126.66
2	AB3	18443.151	107.82
3	AB28	15110.998	88.34
4	AB32	17887.2213	104.57
5	AB43	22276.4926	130.23
6	AB59	13415.8436	78.43
7	AB64	15554.0311	90.93
8	AB97	23721.9074	169.33

*****Each value is an average of three replicates of isolated strains.

3.3.3 PCR amplification and sequence analysis of the Gt-amy

PCR method indicated that ~400 bp amplicon was obtained using the primers GT-intF and GT-intR. Its sequence displayed 99% homology with α-amylases of *Geobacillus* spp. and *G. stearothermophilus*. Using the end regions of this amplicon, and the end regions of the nucleotide sequences of *G. stearothermophilus* α-amylases, internal (intFI and intRI) and external (extFI and extRI) primers had been designed. These amplicons

were sequenced and then, all these sequences were overlapped to obtain the full-lenGth gene, as shown in figure (4.4).

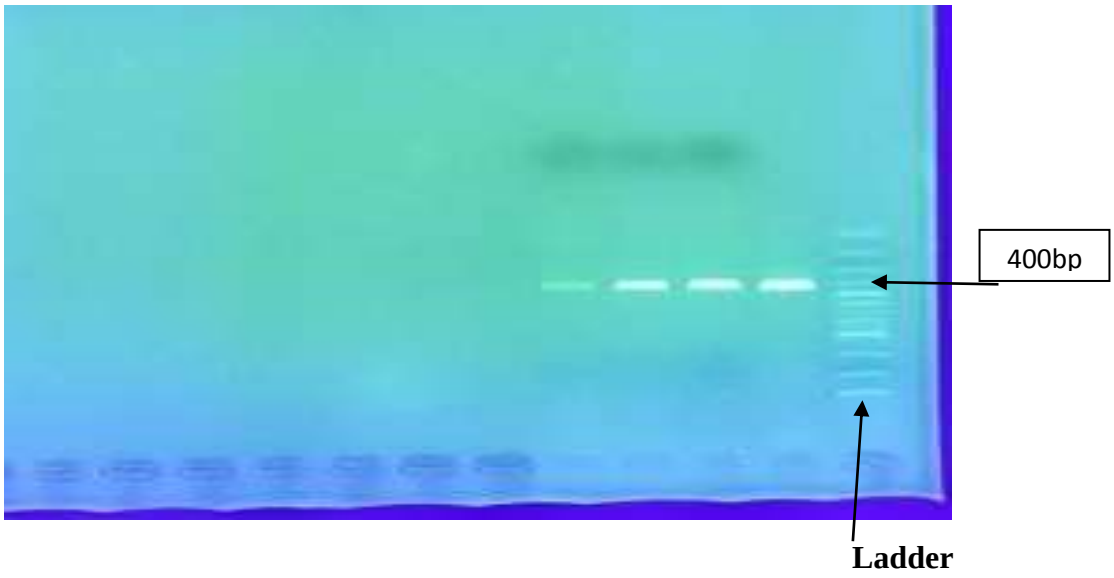

Figure (4.4) PCR products on agarose gel electrophoresis for the detection of *Gt-amy*. L: 400 bp DNA ladder.

Similar to our study, (Deepika and Satyanarayana 2013) used *G. sterothermophilus* for the detection of isolates a gene that can produce amylase enzyme (*Gt-amy*) (19).

3.4 Amylase production using agricultural wastes:

Agricultural wastes are becoming an increasing environmental problem; therefore, the current study was focusing on using different wastes as available and cheap materials to produce amylase. The three isolates AB1, AB43 and AB97 were selected as a good isolate to produce amylase enzyme by using four agricultural wastes as starchy materials in a liquid production medium show in figure (4.5). Results in Table (4.9) investigated that all three isolates could grow and amylase production in a liquid medium with

different types of agriculture wastes. Moreover, broken rice and Potato peel were the best wastes that stimulated and gave the highest enzyme productivity in all isolates compared with the medium have 1% soluble starch, whereas other wastes gave decreased amylase productivity than the standard medium. Also, the results illustrated that the highest amount of amylase production (187.2 unit\ml) was recorded by *G.*

*stearothermophilus AB*43 when used potato peel as substrate, while the lowest value in amylase production was obtained by AB97 in a medium supplemented with 1% wheat bran.

Table 4.6 Amylase production byAB1, AB43 and AB97 isolates using 1% of five different agricultural wastes as the carbon source in a liquid medium.

No.	Agriculture wastes	Enzyme activity (Unit\ ml)		
		AB1	AB43	AB97
1	Wheat bran	133.1	139.8	117.8
2	Broken rice	156.9	183.4	147.2
3	Banana peel	144.6	169.2	130.4
4	Potato peel	159.3	187.2	151.6
5	Positive control (1% starch)	126	130.23	169.33

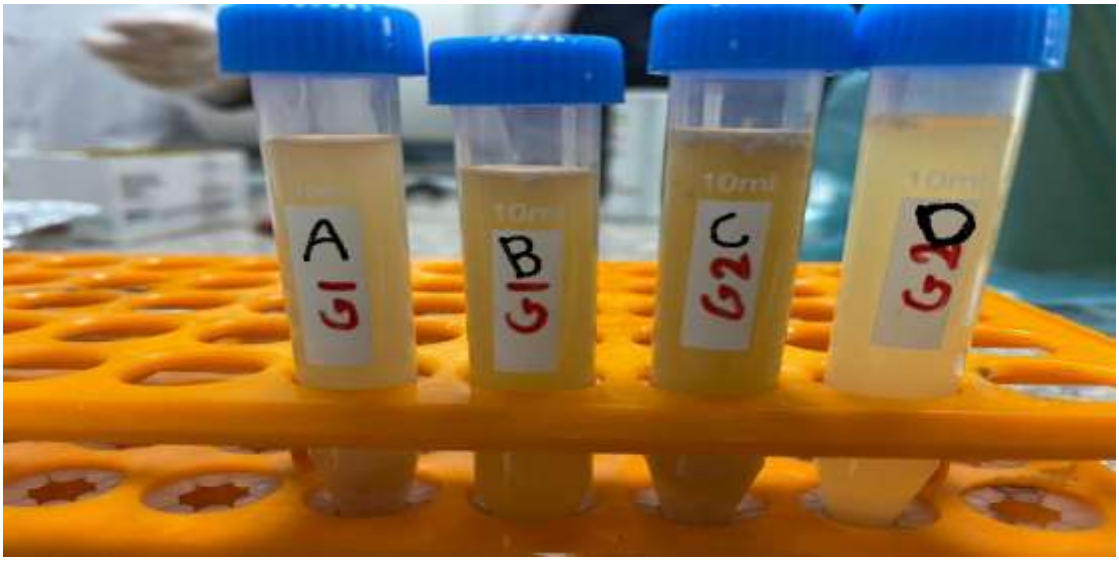

Figure (4.5) Show (A) Wheat bran (B) Banana peel (C) Potato peel (D)Broking rice.

The contents of synthetic media are very expensive and these contents might be replaced with more economically available agricultural by-products to reduce the cost of the media (11). Therefore agro-industrial wastes and by-products such as starchy materials had been used for the Biosynthesis of amylases to solve the pollution problems and obtain a low-cost media (1;14). The use of agricultural wastes makes solid--state fermentation (SSF) an attractive alternative method (7).

Conclusion

Bacterial strains isolated from the soil of Kurdistan-Iraq were isolated and characterized for production of the α-amylase enzyme, Bacterial identification was carried out through morphological and biochemical methods and confirmed via **VITEK® 2 Compact BCL KIT** method as G. stearothermophilus. Distinguishable capability for producing α-amylase in a different method and determining quantitative of enzyme and detection of *Gt-amy* by gel electrophoreses PCR. also using several food wastes as a substrate for producing enzyme. The results of this study suggest the importance of local isolate of *G. stearothermophilus* because these bacteria are very efficient to produce the thermostable industrial enzymes Also, this study indicates the importance of using the food waste fermentation technique, since it is very simple in application, obtaining high yield products, less energy and aeration requirements in comparison with liquid state fermentation.

Reference:

1.Anto, H., Trivedi, U. and Patel, K., 2006. α-Amylase production by Bacillus cereus MTCC 1305 using solid-state fermentation. *Food Technology and Biotechnology*, *44*(2), pp.241-245.

2.Baker, R.T.K., Barber, M.A., Harris, P.S., Feates, F.S. and Waite, R.J., 1972. Nucleation and growth of carbon deposits from the nickel catalyzed decomposition of acetylene. *Journal of catalysis*, *26*(1), pp.51-62.

3.Baris, Nezihi, Ebru Özpelit, Nazile Bilgin Dogan, Hande Kangül, Sefa Gül, Bahri Akdeniz, and Sema Güneri. "The effects of chronic usage of enzyme inhibitors and angiotensin receptor blockers on contrast-induced nephropathy in low-risk patients/Düsük riskli hastalarda anjiyotensin dönüstürücü enzim inhibitörleri ve anjiyotensin reseptör blokerlerinin kronik kullaniminin kontrast madde nefropatisi üzerine etkileri." *The Anatolian Journal of Cardiology* 13, no. 3 (2013): 245.

4.Buck, J.D., 1982. Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Applied and Environmental Microbiology*, *44*(4), pp.992-993.

5.De Bartolomeo, A., Trotta, F., La Rosa, F., Saltalamacchia, G. and Mastrandrea, V., 1991. Numerical analysis and DNA base compositions of some thermophilic Bacillus species. *International Journal of Systematic and Evolutionary Microbiology*, *41*(4), pp.502-509.

6.Egelseer, E., Schocher, I., Sára, M. and Sleytr, U.B., 1995. The S-layer from Bacillus stearothermophilus DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. *Journal of Bacteriology*, *177*(6), pp.1444-1451.

7.Ellaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P. and Srinivasulu, B., 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated Aspergillus species. *Process Biochemistry*, *38*(4), pp.615-620.

8.Glazer, A.N. and Nikaido, H., 2007. *Microbial biotechnology: fundamentals of applied microbiology*. Cambridge University Press.

9.Gusakov, A.V., Kondratyeva, E.G. and Sinitsyn, A.P., 2011. Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. *International journal of analytical chemistry*, *2011*.

10.Halket, G., Dinsdale, A.E. and Logan, N.A., 2010. Evaluation of the VITEK2 BCL card for identification of Bacillus species and other aerobic endosporeformers. *Letters in applied microbiology*, *50*(1), pp.120-126.

11.Haq, I.U., Ali, S. and Iqbal, J., 2003. Direct production of citric acid from raw starch by Aspergillus niger. *Process Biochemistry*, *38*(6), pp.921-924.

12.Ikari, J., Smith, L.M., Nelson, A.J., Iwasawa, S., Gunji, Y., Farid, M., Wang, X., Basma, H., Feghali-Bostwick, C., Liu, X. and DeMeo, D.L., 2015. Effect of culture conditions on microRNA expression in primary adult control and COPD lung fibroblasts in vitro. *In vitro Cellular & Developmental Biology-Animal*, *51*(4), pp.390-399.

13.Joshi, R.S., Jagdale, S.S., Bansode, S.B., Shankar, S.S., Tellis, M.B., Pandya, V.K., Chugh, A., Giri, A.P. and Kulkarni, M.J., 2021. Discovery of potential multi-target-directed ligands by targeting host-specific SARS-CoV-2 structurally conserved main protease. *Journal of Biomolecular Structure and Dynamics*, *39*(9), pp.3099-3114.

14.Kumari, M., Mukherjee, A. and Chandrasekaran, N., 2009. Genotoxicity of silver nanoparticles in Allium cepa. *Science of the total environment*, *407*(19), pp.5243-5246.

15.LARSON, R. and SCHERB, R.E., 1953. Coccidioidal pericarditis. *Circulation*, *7*(2), pp.211-217.

16.Maier, R.M. and Soberon-Chavez, G., 2000. Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications. *Applied microbiology and biotechnology*, *54*(5), pp.625-633.

17.Meena, R.K., Singh, R.K., Singh, N.P., Meena, S.K. and Meena, V.S., 2015. Isolation of low temperature surviving plant growth–promoting rhizobacteria (PGPR) from pea (Pisum sativum L.) and documentation of their plant growth promoting traits. *Biocatalysis and agricultural biotechnology*, *4*(4), pp.806-811.

18.Mehta, D. and Satyanarayana, T., 2013. Biochemical and molecular characterization of recombinant acidic and thermostable raw-starch hydrolysing α-amylase from an extreme thermophile Geobacillus thermoleovorans. *Journal of Molecular Catalysis B: Enzymatic*, *85*, pp.229-238.

19.Mehta, D. and Satyanarayana, T., 2013. Biochemical and molecular characterization of

recombinant acidic and thermostable raw-starch hydrolysing α-amylase from an extreme thermophile Geobacillus thermoleovorans. *Journal of Molecular Catalysis B: Enzymatic*, *85*, pp.229-238.

20.Mielenz, J.R., 1983. Bacillus stearothermophilus contains a plasmid-borne gene for alpha-amylase. *Proceedings of the National Academy of Sciences*, *80*(19), pp.5975-5979.

21.Ou, M.S., Mohammed, N., Ingram, L.O. and Shanmugam, K.T., 2009. Thermophilic Bacillus coagulans requires less cellulases for simultaneous saccharification and fermentation of cellulose to products than mesophilic microbial biocatalysts. *Applied biochemistry and biotechnology*, *155*(1), pp.76-82.

22.Outtrup, H. and Jørgensen, S.T., 2002. The importance of Bacillus species in the production of industrial enzymes. *Applications and systematics of Bacillus and relatives*, pp.206-218.

23.Pandy, M.G. and Anderson, F.C., 2000. Dynamic simulation of human movement using large-scale models of the body. *Phonetica*, *57*(2-4), pp.219-228.

24.Parry, J.M., Turnbull, P.C.B. and Gibson, J.R., 1983. *A colour atlas of Bacillus species*. Wolfe Medical Publications Ltd.

25.Pranay, K., Padmadeo, S.R., Jha, V. and Prasad, B., 2019. Screening and identification of amylase producing strains of Bacillus. *Journal of Applied Biology and Biotechnology*, *7*(4), pp.5-2.

26.Sidkey, N.M., Abo-Shadi, M., Al-Mutrafy, A.M., Sefergy, F. and Al-Reheily, N., 2010. Screening of microorganisms isolated from some enviro-agro-industrial wastes in Saudi Arabia for amylase production. *Journal of American Science*, *6*(10), pp.926-939.

27.Singh Bains, P., Sidhu, S.S. and Payal, H.S., 2018. Investigation of magnetic field-assisted EDM of composites. *Materials and Manufacturing Processes*, *33*(6), pp.670-675.

28.Tarique, A.A., Logan, J., Thomas, E., Holt, P.G., Sly, P.D. and Fantino, E., 2015. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *American journal of respiratory cell and molecular biology*, *53*(5), pp.676-688.

29.Tiwari, V., Drago, M., Frolov, V., Klimenko, S., Mitselmakher, G., Necula, V., Prodi, G., Re, V., Salemi, F., Vedovato, G. and Yakushin, I., 2015. Regression of environmental noise in LIGO data. *Classical and Quantum Gravity*, *32*(16), p.165014.

30.Tural, B., Ertaş, E., Enez, B., Fincan, S.A. and Tural, S., 2017. Preparation and characterization of a novel magnetic biosorbent functionalized with biomass of Bacillus Subtilis: Kinetic and isotherm studies of biosorption processes in the removal of Methylene Blue. *Journal of Environmental Chemical Engineering*, *5*(5), pp.4795-4802.

31.Vaseekaran, S., Balakumar, S. and Arasaratnam, V., 2010. Isolation and identification of a bacterial strain producing thermostable alpha-Amylase.

32.Ventosa, A., Garcia, M.T., Kamekura, M., Onishi, H. and Ruiz-Berraquero, F., 1989. Bacillus halophilus sp. nov., a moderately halophilic Bacillus species. *Systematic and Applied Microbiology*, *12*(2), pp.162-166.

33.Wang, L., Brock, A., Herberich, B. and Schultz, P.G., 2001. Expanding the genetic code of Escherichia coli. *Science*, *292*(5516), pp.498-500.