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ISOLATION AND IDENTIFICATION OF *BACILLUS* SP. PRODUCING OF AMYLASE FROM DIFFERENT SOURCES IN KIRKUK, IRAQ

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Abstract

Thirty bacterial isolates belonging to *Bacillus* spp. obtained from different local sources in Kirkuk-Iraq were screened for their capabilities for producing Amylase. It was found that the isolates designated (5M, 8M, 9M, 12M, 13M, 17M, 18M, 24M, 27M, 30M) have higher capability for producing Amylase. All isolates subjected to secondary screening. The Isolate (13M) was found to be the highest producers with enzyme activity of (6.329U/ml). The identification tests of this isolate were carried out by studying the morphological, microscopic characteristics and biochemical test using Vitek2 compact system. The tests results were confirmed by identification of 16S rRNA gene using polymerase chain reaction (PCR) and its nitrogen base sequencing. The results revealed that this isolate belongs to *Bacillus subtilis*. Recorded in gene bank under the ACCESSION: MN744663.

Key words: Amylase, identification, 16S rRNA, *Bacillus subtilis*, vitek2 compact system.

Introduction

Amylases are enzymes that hydrolyze starch α-1,4 glycoside linkages to give oligosaccharides, maltose and dextrin or ultimately glucose. The starch hydrolytic amylases enzyme commission is EC 3.2.1.1 (Souza and Magalhaes, 2010). Although amylases are produced from different sources (microorganisms, plants and animals), but the one which is produced from microbes are the most suitable for industrial production due to microbial short growth period, low cost effective production, eco-friendly behavior, less handling issues for workers, productivity and easy manipulation of bacterial genes. In industries alpha amylase (microbial enzymes) is of great importance which occupies at the market approximately at about 25% (Gurung *et al*., 2013). The genus *Bacillus* is a major producer of many extracellular enzymes and a common bacterial source for industrial amylase production. However, different strains have different optimal growth conditions and enzymatic production profile for better stability, high capacity and reduced cost of production (Adrio and Demain, 2014, John and Elangovan, 2013) The extremely useful amylase *Bacillus* spp. are known for their antagonistic action against potential harmful fungi and their mycotoxin products which are secondary metabolites obtained from biosynthetic path of certain fungi and they are group of toxic substances that possess carcinogenicity, neurotoxicity, teratogenicity and immunotoxicity prosperities (FAO, 2001, Petzinger and Ziegler, 2000). Although the growth conditions are similar for different species of *Bacillus* genus, they differ in the production of amylases because it depends on the components of the medium and different treatments. In addition, the type of strain where the production of enzymes can be improved by modifying the components of the medium along with other treatments such as case fermentation Liquid, which favors solid-state fermentation in the development of bacteria to produce the enzyme due to the ease of enzyme purification of the products obtained as well as the ease of management of the laboratory process (Dash *et al*., 2015, Abd-Elhalem *et al*., 2015).

This study aimed to isolate bacteria that can produce amylase enzyme obtained from different sources in Kirkuk-Iraq and identify the best producer by Vitek2 compact system and 16S rRNA nitrogen base sequencing.

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Materials and Methods

Sources of Isolation

Soil samples were obtained from different areas Kirkuk. Food materials were obtained from different restaurants and included cooked rice, starch and some other foods.

Primary screening

Ten grams of each sample was suspended in phosphate buffer (pH 7.0) and the suspension was treated at 80°C for 10 minutes to kill the vegetative forms of the bacteria. One ml of this suspension added to 20ml of melted nutrient agar in Petri-dish and incubated at 37°C for 24 hours. The colonies were selected depending on *Bacillus* sp. morphological characteristics. The isolates then screened for amylolytic properties by streaking on nutrient agar plates containing 1% soluble starch at 37°C for 48 hours (Teodoro and Martins, 2000). Amylase activity was detected by the formation of a clear zone around the colonies, when flooded with Gram's iodine solution (1% iodine in 2% potassium iodide). The index of amylolytic activity was resolved according to the method of (Hankin and Anagnostakis, 1975) using the following formula:

Table 1: McFarland standard scale value.

Number of cell × 10^8/ml	Absorbance at 600nm	H_2SO_4 1% (ml)	$BaCl_2.2H_2O$ 1% (ml)	Tube No.
0	0	10	0	1
1.5	0.135	9.95	0.05	2
3.0	0.248	9.90	0.10	3
6.0	0.527	9.80	0.20	4
9.0	0.809	9.70	0.30	5
12.0	1.058	9.60	0.40	6
15.0	1.218	9.50	0.50	7
18.0	1.543	9.40	0.60	8

* Absorption results are adjusted for two replicates.

Table 2: The primer used to amplify the 16S rRNA gene.

Number of nitrogenous bases	Sequences	primers
17	5- CGGGTGAGTAACACGTG 3-	Forward 104F
17	5- CGGTGTGTACAAGGCCC 3-	Reverse1390R

Table 3: Additives in the Eppendorf tube container to premix to amplify the 16S rRNA gene using technique (PCR).

Volume (µl)	Ingredients	S. No.
5	DNA extract	1
2	Forward primer at a concentration of 10 (µM)	2
2	Reverse primer at a concentration of 10 (µM)	3
11	Free nuclease water	4
20	Total volume	

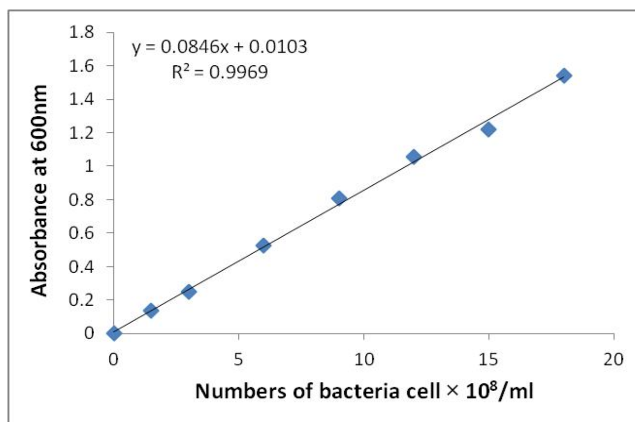

Fig. 1: McFarland standard curve for calculating bacterial cell numbers/ml.

Index of amylolytic activity = *The diameter of the clear zone (Zone) cm / The diameter of the culture zone (Growth) cm*

McFarland stander curve

1907, McFarland established a runs of Barium chloride solutions to near the numbers of bacteria in solutions of comparable turbidity, as determined by plate counts (McFarland, 1907, Collee *et al*., 1996).

These volumes shown in (Table 1) from Barium chloride dihydrate in test tubes and completed to 10ml. The absorbance was read at 600 nm by spectrophotometer for all test tubes. The Blank attended by using Sulfuric acid 1% only. The McFarland stander curve as in (Fig. 1). Using in Approximate the numbers of bacteria cell in solutions/ml. To determine the volume of vaccine was transferred for production medium.

Secondary screening/Enzyme production

A loopful of the bacteria (isolates) was activated in nutrient broth for 24 hours in 37°C and the density of bacteria growth were estimated using McFarland stander carve. After that 1ml (1×10^8 cells/ml) of bacterial suspension was transferred to Starch broth (submerged

Table 4: Conditions approved in the 16S rRNA gene amplification reaction programmed in a (PCR).

Step	The steps	Temperature (°C)	Time (min)	Number of cycle
1	Denaturation	94	5	1
2	Denaturation	94	1	30
3	Annealing	55	1	
4	Extension	72	1:40	
5	Final extension	72	5	1
6	Cooling	4	10	1

At the end of the reaction, 10 (µl) of the 16S rRNA amplified was taken for electrophoresis.

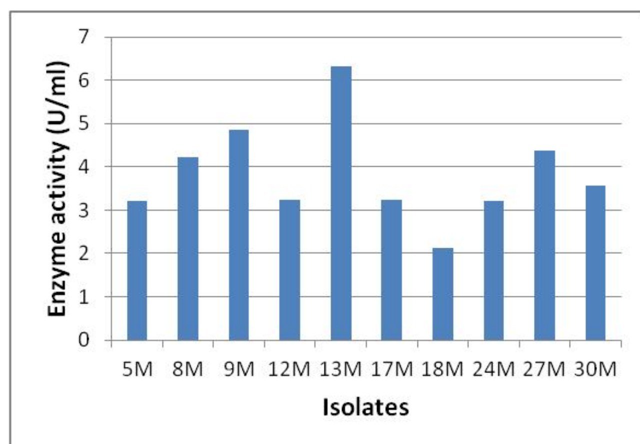

Fig. 2: Enzyme activity (U/ml) of ten isolates selected from Primary screening (Identification of isolates (13M).

culture containing: starch 10g, peptone 5.0g, $MgSo_4$, $7H_2O$ 0.5g and KCl_2, $4H_20$ 0.5g in Distilled water up to1000 ml mark) (Oyeleke *et al*., 2010). The pH of the medium was adapt to pH 7.5 and 50ml was distributed in each Erlenmeyer flasks with a capacity of 300ml and placed in incubator shaker with a speed 150 rpm at 37°C for 72 hours. The growth cultures were filtered by centrifuged at 10000 rpm for 20min at 4°C and the clear supernatant as the crude enzyme.

Determination of Enzyme Assay

Amylase assay in crude enzyme was determined according to Lin *et al*., (1997). The assay reaction mix contained 2ml of a solution of 1% starch in 50 ml sodium phosphate buffer (pH 7.0) and 0.1 ml crude enzyme. Incubation at 40°C 10 minutes, the reaction was stopped by adding 2ml of 3,5 Dinitro Salicylic acid reagents (DNS) and the tubes were heated at 100°C for 5 minutes. The absorbance was consistent by spectrophotometer at 540nm against a blank containing buffer instead of the culture supernatant. Another blank was prepared using part of the supernatant which was heated at 100°C for 5 minutes and used in the reaction in place of buffer. No difference in absorbance between the two blanks was observed. The amount of reducing sugars was calculated from a standard curve constructed by using glucose. One enzyme (unit/ml) is defined as the amount of enzyme that catalyzes the liberation of 1µml glucose per minute under the assay conditions.

Determination of Proteins

Protein was determined using the dye-binding method of Bradford, (1976). Commassie Brilliant Blue G250 (100mg) was dissolved in 500 ml of 95% ethanol. To this solution 100ml of 85% (w/v) phosphoric acid was added and the mixture was diluted with distilled water to a final volume of 1 liter. This solution was then filtered and kept in a dark bottle. 1 ml of Commassie Brilliant Blue G250 solution was added to 0.1 ml enzyme sample. After mixing the absorbance wasread at 595nm after two minutes and before one hour against a blank prepared using 0.1 ml of phosphate buffer and 1 ml of the Commassie Brilliant Blue G250 reagent. A standard curve was built up by using Bovinserum albumin (BSA).

Identification of isolates

Vitek2 compact system Identification: Vitek 2 compact system for identification of the isolate (13M) was achieved by a special diagnostic kit for the Bacillaceae family (BCL), which contains 46 biochemical tests. The procedure was followed according to the instructions of the manufacturer BioMerieux.

Table 5: The diameter of clear zones by different bacterial Isolates on starch agar medium.

Isolates sources	Bacterial Isolates	Index of Amylolytic Activity in (cm)	Isolates sources	Bacterial Isolates	Index of Amylolytic Activity in (cm)
Soil	M1	1.50	Food	M16	1.00
Soil	M2	1.90	Food	M17	2.10
Soil	M3	0.81	Food	M18	2.30
Soil	M4	0.29	Food	M19	0.15
Soil	M5	2.45	Food	M20	0.65
Soil	M6	1.80	Food	M21	1.87
Soil	M7	1.85	Food	M22	1.22
Soil	M8	2.10	Food	M23	1.40
Soil	M9	3.50	Food	M24	3.21
Soil	M10	1.10	Food	M25	1.22
Soil	M11	0.88	Soil	M26	0.98
Soil	M12	3.12	Soil	M27	3.32
Soil	M13	4.50	Soil	M28	1.25
Soil	M14	0.76	Soil	M29	1.43
Soil	M15	0.31	Soil	M30	2.45

Molecular Identification

The isolate (13M) was done by sequencing of 16S rRNA gene technique following these steps:

DNA Extraction

The bacterial DNA was extracted by Presto Mini gDNA Bacteria Kit from Taiwan's Geneaid Company.

DNA Amplification

The Polymerase chain reaction (PCR) technique was used to amplify the 16S rRNA gene of the isolate selected using the following primers as mentioned in (Sacchi *et al*., 2002).

Table 6: The result of Biochemical test for *Bacillus subtilis* 13M by Vitek2 compact system.

BioMerieux Customer — Laboratory Report — Printed Nov 12. 2018 12:25 CST

System #: — Printed by: lab admin

Isolate Group: 11-1

Card Type: BCL Testing Instrument 0000148FEEC5 (VITEK2C)

Bionumber: 2225101100456621

Organism Quantity:

Comments:	

Identification Information	Card: BCL	Lot Number: 2390084203	Expires: Feb 18. 2022 12:00
	Completed: Nov 12. 2018 00:33 CST	State: Final	Analysis Time: 14:25 hours
Selected Organism	96% Probability Bacillus subtilis Bionumber: 2225101100456621		Confidence: Excellent Identification

Biochemical Details																	
1	BXYL	+	3	LysA	-	4	AspA	(+)	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	-	13	TyrA	+	14	BNAG	-
15	APPA	-	18	APPA	-	19	CDEX	-	21	dGAL	-	22	INO	+	24	MdG	+
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	(+)	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	+	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl6.5%	+	59	KAN	-
60	OLD	-	61	ESC	+	62	TTZ	-	63	POLYB_R	-						

The polymerase chain reaction was done in a mixture as revealed (Table 3) in PCR thermal cycler. The device was programmed according to (Table 4).

Electrophoresis of PCR products and Agarose gel preparation

The gel at a concentration of 1% was prepared by dissolving 0.5g of Agarose in 50ml solution 1X TBE. Heated by microwave oven for 2 min, leaved to cool at 55-60°C and 2µl of Ethidium bromide was added to the cooled Agarose solution into the prepared gel-casting tray. After solidification of the gel and removing of the caps and comb carefully, gel (on the tray) Placed into electrophoresis chamber and completely covered with 1XTBE electrophoresis buffer. The DNA samples and the ladder into wells in consecutive order were loaded.

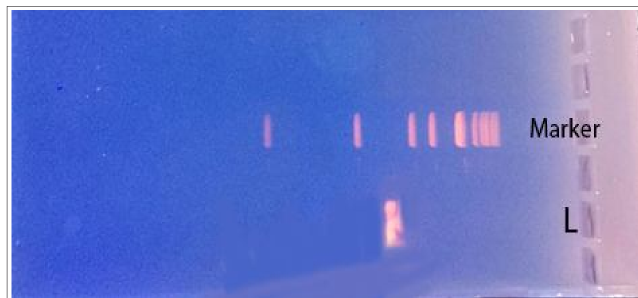

Fig. 3: Gel Electrophoresis Agarose, L: Amplification of gene 16S rRNA for isolate, *B. subtilis* 13M Marker : 500-10200 bp.

The ladder supplied from the Promega Company (Pulimamidi and Nomula, 2012).

Results and Discussion

Isolation and primary screening

After performing serial dilution method, numerous colonies were obtained from different samples from soil and foods. Forty five colonies were grown in nutrient agar plates containing 1% soluble starch and out of Forty five colonies, thirty colonies showed clear zone around them and thus it shows the existence of bacteria producing amylase (Table 5) when treated with iodine as described in the materials and methods piece. Ten isolates from various foods sources and twenty from different soils.

Secondary screening

Submerged fermentation was accomplish for production of amylase as reported earlier Among thirty tested bacterial isolates, ten amylolytic bacterial strains (top amylase producer) namely (5M, 8M, 9M, 12M, 13M, 17M, 18M, 24M, 27M, 30M) were selected for further investigations for subjected to Secondary screening by assay enzyme activity (U/ml). The isolate 13M In secondary screening showed maximum activity 6.329 U/ml (Fig. 2) followed by 9M (4.843 U/ml) and 27M (4.376 U/ml). Minimum activity was found in 18M (2.121 U/ml). Established on secondary screening 13M isolate was selected as best producer of amylase and subjected to biochemical tests and molecular identification. Some studies have indicated the successful use of the reagent method now primary and secondary screening to isolate the microorganism species producing Amylase. Oseni and Ekperigin, (2013) managed isolated eight different bacteria species from forest soil producing Alpha amylase. Another study done by Wanjare *et al*., (2013) among four bacterial isolates from sewage soil source, one isolate maximum produced of starch hydrolysis (clear zone). This isolate was identified as *Bacillus* sp. and Verma *et al*., (2011) found the different species of *Bacillus* producing amylase from waste potato dumpsite source.

Vitek2 compact system Identification

The isolate (13M) was identified by using Vitek2 compact system and the result showed similarity of this isolate to *Bacillus subtilius* by a probability of 96% as shown in (Table 6).

Table 7: Sequences of 16S rRNA gene for isolate (13M).

No. of nitrogen bases	Sequences of nitrogen base for 16S rRNA gene
1143	TTTGACTGGGCTACTCCGGGAACCGGGGCTAATACCGGATGGT TGTTTGAACCGCAGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTAC AGATGGACCCGCGGCGCATAGCTAGTTGGTGAGGTAACGGCTCACCAAGG CAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGAT CGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGT ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG GGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCG GGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGA ATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGG CGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGG GAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAG CACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA AGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGT CCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG TTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGG AGGAAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT ACACACGTGCTACAATGGACAGAACAAAGGGCACCGAAACCCCGAGGTTA

Molecular Identification

DNA extraction

The DNA was extracted from *B. subtilius* 13M and the purity of DNA were examined by Nano Drop 260-280nm absorbance with a purity of 1.99 which is adequate for Polymerase Chain Reaction (PCR) process. Reported that the PCR did not need a large quantity of DNA which may instead produce unlimited amplifying products. On other hand, an adequate quantity of DNA may reduce the accuracy.

Table 8: Identity percentage of 16S rRNA gene nitrogen bases sequences of NCBI strains with the sequencing of *Bacillus subtilis* 13M.

	Strain	Identity %	Accession
1	*Bacillus subtilis* J732	99	MK729018.1
2	*Bacillus subtilis* LJFMLL6	99	MK086953.1
3	*Bacillus subtilis* TCCC11212	99	FJ393297.1
4	*Bacillus subtilis* IICTSVMH13	99	FR849706.1
5	*Bacillus subtilis* VBN25	99	MG027675.1
6	*Bacillus subtilis* af-B8	99	MN512303.1
7	*Bacillus subtilis* JYM31	99	MN511798.1
8	*Bacillus subtilis* SR3-30	99	MN421487.1
9	*Bacillus subtilis* SR2-27	99	MN421466.1
10	*Bacillus subtilis* SR3-4	99	MN421473.1

Polymerase Chain Reaction (PCR)

Results of the gel electrophoresis Agarose show by using U.V light detector of DNA extracted from isolate 13M by PCR amplification of gene 16S rRNA assay were shown in (Fig. 3).

Sequence analysis

PCR products were sent to Macrogen Company/ Korean to determine the sequences of the nitrogen bases and the result showed the molecular size of gene amplification band was 1143bp (Table 7) comparing with ladder size at the same conditions. These sequences were compared with the available information on those found in the NCBI through the PLAST Nucleotide program. Identified isolation shown there is a 99% match between this isolate and the strains of *Bacillus subtilis* bacteria registered in NCBI as shown in the (Table 8). The finally identified isolated strain was *Bacillus subtilis* 13M, founded on the results of the morphological, observation, physiological and biochemical tests, as well as the 16S rRNA sequence analysis. The nitrogen bases sequences from *Bacillus subtilis* 13M registered on the NCBI which belong to *Bacillus subtilis* strain 13M ACCESSION: MN744663. Hasan *et al*., (2017) they managed that the molecular size gene of 16S rRNA isolated was 1082 base pairs. While Dash and Rehman (2015) found that the

isolated *Bacillus subtilis* BI19 molecular size for gene 16S rRNA of bacteria has reached 500 bp.

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