PURIFICATION, CHARACTERIZATION AND SEQUENCING OF AMYLASE A FROM ALKALIPHLIC *BACILLUS LICHENIFORMIS* STRAIN-MK7

Mohamed kasem[1], Aysam M. Fayed[2], Samah Eldemery[2], Adel Okba[1] and Atef Ibrahim[1]

1- Microbial biotechnology dept. 2- Molecular Biology dept., Genetic Eng. and Biotech. Research Inst., University of Sadat City (USC), Egypt

Corresponding author: atef.ibrahim@gebri.usc.edu.eg

ABSTRACT

Two degenerative primers were designated from the closest Bacillus, *Bacillus licheniformis* strain DSM13 (ATCC 14580) which has complete genome to get amylase gene from our *Bacillus licheniformis* strain MK7 (**KP322016**) by PCR technique. We got about 1500 bp PCR, the sequence analysis revealed that the alkaliphilic cellulase belongs to glycosyl hydrolase family 12A which has 100 % identity to glycoside hydrolase from Bacillus sp. (WP009329478) and has 99 % identity to family 12 xyloglucanase from Bacillus licheniformis (2JEMIA). The nucleotide sequence of the alkaliphilic cellulase gene (cel12A) was deposited to the GenBank under accession number (KP322015). The cel12A gene from B. licheniformis strain MK7 has open reading frame of 1500 bp that codes for 449 amino acid which has a molecular weight of 55KDa protein. The enzyme protein was further purified using ammonium sulphate precipitation and chromatography. The characterization of amylase protein showed that the enzyme has an optimum temperature at (37°C), optimum pH (9.0) and Some metals increase the enzyme activity such as $CoCl_2^+$, K^+ and EDTA , but others decrease the activity such as Ag^+, Zn^{2+} and urea ions.

Keywords: Amylase A, Purification, characterization, *Bacillus licheniformis* strain –MK7.

INTRODUCTION

Alkaliphillic bacteria are defined as organisms that grow optimally at alkaline pH, with pH optima for growth being in excess of pH 8 (usually between 8 and 10), and some being capable of growing at pH higher than 11 **(Grant and Jones, 2000; Horikoshi, 1999)**. Alkaliphillic bacteria are reported to be a rich source of alkaline active enzymes *e.g.* amylase, cellulase, xylanase, and other enzymes which have numerous applications in industrial processes (**Horikoshi, 1991**). Among the bacteria, the genus *Bacillus* is an attractive industrial organism due to growth rate, extracellular secretion and biosafety capacity (**Schallmey et al., 2004**). Amylases, glycoside hydrolases, break starch into glucose, maltose, maltotriose and dextrin by hydrolysis of glyosidic bonds. Therefore they are also called digestive enzymes. The first enzyme produced industrially was amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (**Pandey *et al.,* 2000)**. Amylases are obtained from various origins like plant, animal, bacterial and fungal. Several researchers produced amylases enzyme using *Bacillus* sp. **(Yuguo *et al*., 2000; Young *et al*., 2001; Dharani, 2004 and Zambare, 2010a).** Amylase has many applications in food, textile, paper and pulp,

pharmaceuticals, baking and beverages, detergent and leather industries **(Pandey *et al.,* 2000; Reddy *et al*., 2003a and Kar *et al*., 2010).** Amylases constitute a class of industrial enzymes, which alone form approximately 25 % of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals **(Mamo *et al*., 1999; Oudjerouat *et al.,* 2003; Pandey *et al*., 2000)**. Amylases are classified into two categories; Endoamylases and Exoamylases **(Gupta *et al*., 2002)**. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyze from the non-reducing end, successfully resulting in short end products. The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, incubation period, pH, temperature, metal ions and thermos ability **(Pandey *et al*., 2000)**. In fact, such industrially important microorganisms found within the genus *Bacillus*, can be

exploited commercially due to their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the extracellular medium and safe handling **(Pandey *et al*., 2000).** The most widely used thermostable amylases in the starch industry are produced from ***B. licheniformis*** **(Morgan et al., 1981).** Microbial production of amylase is more beneficial than other sources because it is economical; production rate is high and can be engineered to obtain enzymes of desired characteristics. The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries, paper industries etc. With the emergence of biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. These increased uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes **(Aiyer *et al.,* 2005).**

Present study is related to the isolation, characterization and sequencing of amylase producing bacteria from the soil samples collected from the fields of Sadat city, (Menoufiya governerate, Egypt.

MATERIALS AND METHODS

Isolation of bacterial cultures

Soil samples were collected from field of Sadat city (menofya), Egypt from 3 to 4 cm depth then transferred into sterile plastic bags and maintained in aseptic conditions. Isolation of soil bacteria was performed by serial dilution and spread plate method as described earlier **(Aneja, 2003).** A volume of 30µl ml of each dilution was transferred aseptically to starch agar plates. The sample was spread uniformly. The plates containing Starch 0.5 %, Trypton 1.5 %, $Nacl_2$ 0.5 %, Agar 1.5 %, Na2CO3 1 % (w/v), the pH was adjusted to 9.5 with Na_2CO3 (10%, w/v) then incubated at 37°C for 24 hr. The bacterial isolates were further sub cultured to obtain pure culture. Pure isolates on starch agar slants were maintained at 4ºC.

Genomic DNA isolation

Genomic DNA of the two amylolytic *Bacillus licheniformis* strain-mk7 was extracted from cells grown on LB broth according to the protocol for bacterial DNA extraction with the Gene JET Genomic DNA Purification Kit (#K0721) provided by Thermos Scientific.

PCR amplification of alkaliphilic amylase gene

Genomic DNA fragment encoding amylase (AmyA) was amplified by PCR with chromosomal DNA of *Bacillus licheniformis* strain-MK7 as a template and two degenerative oligonucleotide primers: Ksm-F GGATCCATGAAACAACAAAAACGGCTTTAC and Ksm-R1-GGATCCCTATCTTTGAACATAAATTGAAAC and Ksm-R2 – GGATCCTTCTGCTGCGACATCAGGATGGTC were synthesized based on the nucleotide sequence of closest *Bacillus licheniformis* strain DSM13 (ATCC 14580) amylase.

DNA Sequencing of amylase gene

Nucleotide sequencing was carried out on applied Bio systems model 373 A (Lincoln, Nebr) model 4000L automated DNA sequencer with appropriate dye primers (Macrogen Inc. Souel, Korea). The nucleotide sequence data was analyzed with GENETYX computer software (Software Development Co. ltd., Tokyo, Japan). Homology searches in Gene Bank were carried out with a Blast Program **(Kenji *et al.,* 1996).**

Enzyme assay

The isolate which showed high zone of clearance were used for further studies. Freshly prepared inoculum was used to inoculate the production medium.500 ml of the production medium was inoculated with 10 µl of bacterial inoculum. The flask was loaded on a rotary shaker incubator at a speed of 2000 rpm at 37°C for 24 hours. After incubation, broth was centrifuged at 10000 rpm for 15 min was collected and used for the estimation of amylase. Purification of amylase enzyme was achieved by ammonium sulphate precipitation followed by dialysis. 100 ml of cell free extract was saturated with ammonium sulphate up to 80%. The content was incubated overnight and centrifuged at 6000 rpm for 20 min Amylase was determined by spectrophotometric method as described by **Fisher and Stein (1961)**. According to the procedure 1.0ml of culture extract 'enzyme' into a test tube and 1ml of 1% soluble starch in phosphate buffer (pH 9.0) was added in test tube. The test tubes were covered and incubate at 35°C for 15 minutes in water bath. Then 2.0 ml DNS reagent was added in each tube to stop the reaction and kept in boiling water bath for 5 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer **(Fisher and Stein, 1961).**

Enzyme characterization

Optimum temperature

The activity was determined by carrying out the assay at several temperatures. 100 µl of appropriate concentration of enzyme were added to 100 µl of 1% starch and completed to 1 ml with 800 µl of 20 mM glycine-NaOH buffer (pH 9.0), and incubated at various temperatures (10, 15, 25, 37, 50, and 70) for 30 min. The activity was then measured according to **(Miller, 1959).**

Effect of pH on amylase A activity.

Buffer (0.05M) of different pH ranging from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 9.5, 10.0 were prepared using different buffer system. Each of this buffer solution was used to prepare 1% soluble starch solution used as substrate in assaying the enzyme as the following 100 µl of appropriate concentration of enzyme were added to 100 µl of 1% starch and completed to 1 ml with 800 µl of various buffers then incubation at 37^{o}C for 30 min, add 1 ml of DNS into the mixture. The assay was carried out according to standard assay procedure measured the activity according to (**Miller, 1959**).

Effect of Heavy Metals on Enzyme Activity.

For activators and/or inhibitors sensitivity studies, the enzyme was pre-incubated with a final concentration of 1mM of various reagents (Ag+, K+, Co2+, Fe3+, Mg2+, Cu2+, Zn2+, EDTA, Urea) dissolved in 50 mM glycine-NaOH buffer (pH 9.0) at 37ºC for 30 min. **(Oda et al., 1993)** .The residual activity was measured by using the standard assay conditions.

RESULTS AND DISCUSSION

PCR product of alkaliphilic amylase gene from *B. licheniformis* strain-MK7

PCR amplification was performed as mentioned before (materials and methods) in a final volume 200µl, giving a good band at 1500 bp on agarose gel as shown in figure (xxx).The target band (1500 bp) excised, eluted and purified.

Nucleotide sequence of alkaliphilic amylase gene

The sequence of the obtained clone was determined on both strands by dideoxynucleotide triphosphate (ddNTP) chain termination method of (**Sanger *et al.*, 1977**) using the automated sequence machine at Macrogen, South Korea. The obtained sequence was assembled, analyzed and compared with sequence in nucleotide database (NCBI) using the BLAST algorithm. The entire open reading frame of amylase gene containing 1500 bases encodes a protein with (**449**) amino acids and a molecular mass (**53** KDa) and has been deposited in the GenBank database under accession number (**KT693278**) as shown in figure .

Characterization of alkaliphilic amylase

Optimum temperature: Alkaliphilic amylase from *B. licheniformis strain MK7* has a wide range of activity at different degrees of temperatures, but it has been found that the highest activity for amylase was determined at 37C° which represents the optimum temperature as shown in figure (6).

Optimum pH

Results recorded in figure (7) show that the activity of the alkaliphilic amylase increased as the pH values increased up to pH 9 which represents the optimum pH.

Effect of some metal ions

Data in figure (8) show that amylase activity response to activators and inhibitors. It appeared that $Mg^{2+,}$ K^{+} and EDTA increase the enzyme activity, whereas Ag^{+}, Zn^{2+} and Urea cause the most inhibition of enzyme activity.

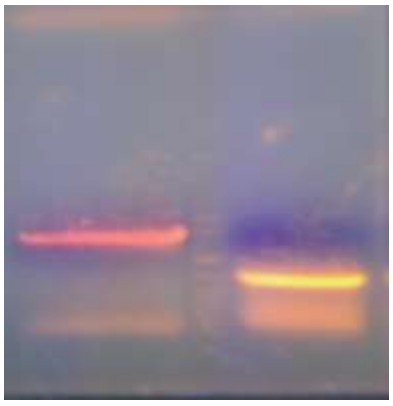
The genus Bacillus produces a large variety of extracellular enzymes, of which amylases are of particularly considerable industrial importance **(Swain *et al.,* 2006)**. Present study deals with the production condition optimization and partial purification of crude extracellular amylase produced by ***Bacillus licheniformis*** was able to hydrolyze starch showing zone of hydrolysis around the colonies on agar medium supplemented with soluble starch four amylotic bacterial colonies among 14 colonies which were positive for amylase production isolated from farm soils of Sadat City, Menofiya government, Egypt. The major factors involved in the biosynthesis of amylases include growth phase, type and concentration of chemicals in the growth media, as well as inducibility and repressibility of the enzyme **(Ikram et al., 2002).**

we designed primers for amylase genes from the genome of the closest strains having complete genome sequence. Amylase gene was amplified by PCR by using A strain of our lab stocks an alkaliphilic *Bacillus licheniformis* strain MK7 which have accession number (KP322016) was used. Three degenerative primers for *Bacillus licheniformis* was used , two primers for the whole amylase gene and two primers for about half the gene, one bands were obtained at the expected size in PCR amplified

(**Fig**.**1**) showing amylotic activity of four bacterial

product of amylase gene from *Bacillus licheniformis* strain MK7 appeared at about 1500 bp on the agrose gel. The target band (1500 bp) excised, eluted, purified and a part was sequenced at Macrogen, South Korea and compared with sequence in nucleotide database (NCBI) using the BLAST algorithm, Phylogenetic tree based on a comparison of the amino acid sequences of amylase *licheniformis* (WP_025807921.1), *Bacillus licheniformis* (ABF61440.1) and *Bacillus licheniformis* (AHJ11193.1).

Figure. (2). Genomic DNA extraction



(**Fig**.**3**) showing PCR products of amylase gene from *Bacillus licheniformis* strain strain mk7 using two pairs of primers; lane 1 the whole amy A gene, l1ne 2, about half of amyA gene, and M 1kb ladder DNA

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          P  H  S  A  A  A  A  A  N  L  N  G  T  L  M  Q  Y  F  E  W  Y  M  P  N  D  G
1         GCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGACGGCC   80

          Q  H  W  K  R  L  Q  N  D  S  A  Y  L  A  E  H  G  I  T  A  V  W  I  P  P  A  Y
81        AACATTGGAAGCGCTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATAT   160

          K  G  T  S  Q  A  D  V  G  Y  G  A  Y  D  L  Y  D  L  G  E  F  H  Q  K  G  T  V
161       AAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGT   240

          R  T  K  Y  G  T  K  G  E  L  Q  S  A  I  K  S  L  H  S  R  D  I  N  V  Y  G
241       TCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGTTTACGGGG   320

          D  V  V  I  N  H  K  G  G  A  D  A  T  E  D  V  T  A  V  E  V  D  P  A  D  R  N
321       ATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAAC   400

          R  V  I  S  G  E  H  R  I  K  A  W  T  H  F  H  F  P  G  R  G  S  T  Y  S  D  F
401       CGCGTAATTTCAGGAGAACACCGAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTT   480

          K  W  H  W  Y  H  F  D  G  T  D  W  D  E  S  R  K  L  N  R  I  Y  K  F  Q  G
481       TAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTTTCAAGGAA   560

          K  A  W  D  W  E  V  S  N  E  N  G  N  Y  D  Y  L  M  Y  A  D  I  D  Y  D  H  P
561       AGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCT   640
561       TCCGAACCCTAACCCTTCAAAGGTTACTTTTGCCGTTGATACTAATAAACTACATACGGCTGTAGCTAATACTGGTAGGA   640

          D  V  A  A  E  I  K  R  W  G  T  W  Y  A  N  E  L  Q  L  D  G  F  R  L  D  A  V
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641     GATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGT   720

          K  H  I  K  F  S  F  L  R  D  W  V  N  H  V  R  E  K  T  G  K  E  M  F  T  V
721     CAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACGGTAG   800

        A  E  Y  W  Q  N  D  L  G  A  L  E  N  Y  L  N  K  T  N  F  N  H  S  V  F  D  V
801     CTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGACGTG   880

         P  L  H  Y  Q  F  H  A  A  S  T  Q  G  G  G  Y  D  M  R  K  L  L  N  S  T  V  V
881     CCGCTTCATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACAGTACGGTCGT   960

          S  K  H  P  L  K  A  V  T  F  V  D  N  H  D  T  Q  P  G  Q  S  L  E  S  T  V
961     TTCCAAGCATCCGTTGAAAGCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACTGTCC   1040

        Q  T  W  F  K  P  L  A  Y  A  F  I  L  T  R  K  S  G  Y  P  H  V  F  S  G  D  M
1041    AAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGAAATCTGGATACCCTCACGTTTTCTCCGGGGATATG   1120

         Y  G  T  K  R  N  S  P  P  E  I  L  A  L  K  H  K  I  E  P  I  L  K  A  R  K  Q
1121    TACGGGACGAAAAGAAACTCCCCCCCCGAAATTCTTGCCTTGAAACACAAAATTGAACCCATCTTAAAAGCGAGAAAACA   1200
1121    ATGCCCTGCTTTTCTTTGAGGGGGGGGCTTTAAGAACGGAACTTTGTGTTTTAACTTGGGTAGAATTTTCGCTCTTTTGT   1200

          Y  A  Y  G  A  Q  H  D  Y  F  D  H  H  D  I  V  G  W  T  R  E  G  D  S  S  V
1201    GTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCGGTTG   1280

        A  N  S  G  L  A  A  L  I  T  D  G  P  G  G  A  K  R  M  Y  V  G  R  Q  N  A  G
1281    CAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCAAAACGCCGGT   1360

         E  T  W  H  D  I  T  G  N  R  S  E  P  V  V  I
1361    GAGACATGGCATGACATTACCGGAAACCGTTCGGAGCCGGTTGTCATC    1408
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Fig 4. The sequence of *acillus .licheniformis* MK7 amyA gene and its deduced amino acid residues (in upper area), with restriction map for some restriction enzymes.

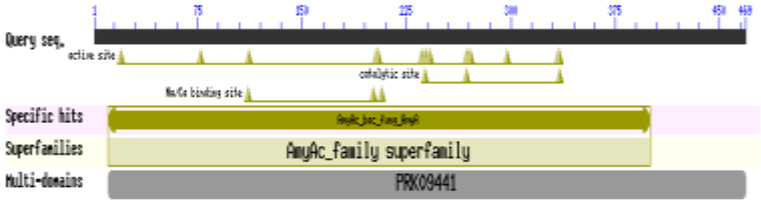

Fig 5. Putative conserved domains have been detected, click on the image below for detailed results.

```
AKI30040.1        ----------------------------------------MKQHKRLYARLLPLLFAL
AJO16877.1        MRRKRHIGAFLLQENIGKTIFAKNSKYLYNSMCFTLKGERKIMKQHKRLYARLLPLLFAL
mk_0000000        ------------------------------------------------------------
ABF61440.1        ----------------------------------------MKQQKRLYARLLPLLFAL
WP-025807921      ----------------------------------------MKQQKRLYARLLTLLFAL
AHJ11193.1        ----------------------------------------MKQQKRLYARLLPLLFAL

AKI30040.1        IFLLPHSAAAAASLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGT
AJO16877.1        IFLLPHSAAAAASLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGT
mk_0000000        ----PHSAAAAANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGT
ABF61440.1        IFLLPHSAAAAANLKGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGT
WP-025807921      IFLLPHSAAAAANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGT
AHJ11193.         IFLLPHSAAAAANLKGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGT
                      ********.*:**********************************************

AKI30040.1        SQDDVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAINSLHSRDINVYGDVVINHKGGA
AJO16877.1|       SQDDVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAINSLHSRDINVYGDVVINHKGGA
mk_0000000        SQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGA
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ABF61440.1        SQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVITNKGGA
WP-025807921      SQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGA
AHJ11193.         SQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGA
                  ************************************:***************..*****

AKI30040.1        FILTREAGYPQIFYGDMYGTKGASQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIV
AJO16877.1        FILTREAGYPQIFYGDMYGTKGASQREIPALKHKIEPILKARKQYAYGAQHDYFDHHNIV
mk_0000000        FILTRKSGYPHVFSGDMYGTKRNSPPEILALKHKIEPILKARKQYAYGAQHDYFDHHDIV
ABF61440.1        FILTRESGYPSGFYGDMYGTKGASQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIV
WP-025807921      FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIV
AHJ11193.         FILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHD--
                  *****::***  * *******  *  ** ****************************:

AKI30040.1        GWTREGDSSVANSGLAALITDGPGGTKRMYVGRQNAGETWHDITGNRSDSVVINAEGWGE
AJO16877.1        GWTREGDSSVANSGLAALITDGPGGTKRMYVGRQNAGETWHDITGNRSDSVVINAEGWGE
mk_0000000        GWTREGDSSVANSGLAALITDGPGGA-RMYVGRQNAGETWHDITGNRSEPVVI-------
ABF61440.1        GWTREGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGE
WP-025807921      GWTREGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGE
AHJ11193.         ------------------------------------------------------------

AKI30040.1         FHVNGGSVSIYVQR
AJO16877.1         FHVNGGSVSIYVQR
mk_0000000         --------------
ABF61440.1|        FHVNGGSVSIYVQR
WP-025807921       FHVNGGSVSIYVQR
AHJ11193.|         --------------
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Fig 6. Multiple deduced amino acid sequence alignment of amylase gene (KT693278) from *B. licheniformis* strain-MK7 with other reported, *Bacillus licheniformis*(AKI30040)*, Bacillus licheniformis*(AJO16877), *Bacillus licheniformis* (WP-025807921),*Bacillus licheniformis* (ABF61440) and *Bacillus licheniformis* (AHJ11193)

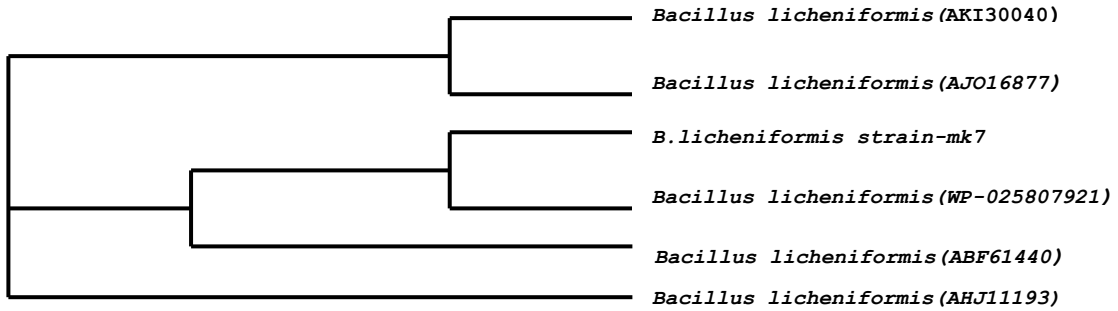
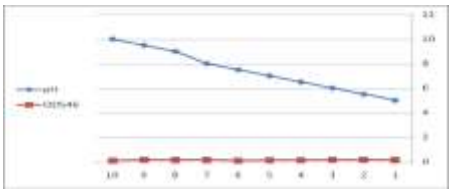
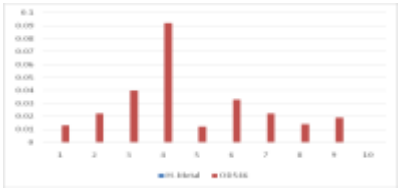


Fig 7. Phylogenetic tree based on a comparison of the amino acid sequences of amylase producing bacterial isolate *B. licheniformis* strain-MK7 and some of their closest phylogenetic relatives, Phylogenetic tree based on a comparison of the amino acid sequences of amylase producing bacterial isolate *B. licheniformis* strain-MK7 and some of their closest phylogenetic relatives *Bacillus licheniformis*(AKI30040)*, Bacillus licheniformis*(AJO16877), *Bacillus licheniformis* (WP-025807921),*Bacillus licheniformis* (ABF61440) and *Bacillus licheniformis* (AHJ11193)



(Fig.8) shows effect of differenet PH on Amylase activity.



(Fig.10) Shows effect of different metal ions on amylase activity**.**

The second part of amylase gene purified was used for cloning the gene in TA cloning, we tried to clone the AmyA several time into two different TA clonning vectors from promega (PGEM) and ferments but there's no gene transfers this may be the longer size of PCR fragment. The amylase gene of *Bacillus licheniformis* strain-mk7 was amplified by PCR technique using consensus primer sequences found in closely related bacteria, the resultant amplicon, did show the presence of a signal sequence.When the sequence was analyzed by the GENETYX,it was predicated that the encoded amylase enzyme could be secreted through the extracellular medium of *lichenniformis* mk7. The amylase gene of *Bacillus licheniformis* strain -mk7 and other amylases from two alkaliphilic amylolytic isolates (ksm1and ksm2) were identified according to the methods in *Bergey's Manual of Systematic Bacteriology* and showed rod-shaped morphology; Gram positive reaction, catalase positive showed that they were long *Bacillus* sp. Sequencing of amylase gene of *Bacillus licheniformis* strain MK7 has been completed by using three degenerative oligonucleotide primers whose sequences were designed on the basis of amylase sequences of *Bacillus licheniformis* strain DSM13 to get the whole gene sequence open reading frame (ORF). The nucleotide sequence of the alkaliphilic amylase gene (AmyA) was deposited to the GenBank under accession number (KT693278). The deduced amino acids sequence derived from the open reading frame of AmyA was analyzed. The mature protein

(Fig.9) shows effect of temperature difference on Amylase activity.

Table 1. Amino acid composition of amylase A of *Bacillus licheniformis* strain-mk7

Amino Acid	Number	Mol%
Ala A	39	8.32
Cys C	0	0.00
Asp D	36	7.68
Glu E	22	4.69
Phe F	19	4.05
Gly G	40	8.53
Ile I	19	4.05
Lys K	29	6.18
Leu L	29	6.18
Met M	7	1.49
Asn N	24	5.12
Pro P	17	3.62
Gln Q	17	3.62
Arg R	21	4.48
Ser S	25	5.33
Thr T	27	5.76
Val V	29	6.18
Trp W	16	3.41
Tyr Y	28	5.97

was 449 amino acids in length and has a calculated molecular mass of 55 KDa. Similarity searches performed with Basic Local Alignment Search Tool (BLAST http://www.ebi.ac.uk/Tools/sss/wublast/) using the deduced amino acid sequence of AmyA as the query

revealed its belonging to family 12A enzymes (http://www.cazy.org/12A.html; extracted from the carbohydrate-active enzymes database (http://www.cazy.org/, **(Cantarel *et al.,* 2009)**. Figure (19) showing effect of temperatures on amylase activity, it was found that enzyme exhibited maximum activity at 37°C. When the temperature increased above 37°C, enzyme activity was affected negatively and gradually reduced. In the same way, when the temperature was below 37°C, a gradual decline in the amylase activity was observed. Various *Bacillus* species are produced amylase enzyme at a wide range of temperatures. *Bacillus amyloliquefaciens*,

Bacillus subtilis, *Bacillus licheniformis an*d *Bacillus stearothermophilus* are among the most commonly used *Bacillus* species reported to produce α-amylase at temperatures of 37-60°C **(Mendu *et al*.,2005b; Mishera *et al*., 2005a; Syu and Chen, 1997;Mielenz, 1983)**. The optimum temperature of the α-amylase is 37^{o}C, with a loss of activity over 70^{o}C. This property might limit industrial applications of the enzyme at high temperatures, but favors its application in processes that require complete inactivation of the enzyme, such as the baking industry **(Coronado *et al*., 2000)**. The broad range of temperatures and the enzyme's high activity at both moderate and lower temperature values make this enzyme highly attractive for both basic research studies and industrial processes. A modern trend among consumers is to use colder temperatures for laundry or dishwashing. At lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with α-amylases working optimally at low or moderate temperatures can overcome this problem (**Van der Maarel *et al*., 2002).**Figure (20) reflects the effect of pH on amylase activity. In fact pH affects the enzyme activity it showed that pH 9 enhance amylase activity to reach its maximum. Therefore we could say that pH 9 is the optimum pH for amylase activity. *B*. *subtilis*, *B*. *licheniformis*, and *B*.*amyloliquefaciens*, like *Bacillus cereus* GUF8**,** require an initial pH of 7.0 for maximal amylase production **(Haq *et al*., 2005a; Tanyildizi, *et al*., 2005b; Syu and Chen, 1997).** The archaeal pullulanases reported to date show maximal activity in the pH range 5.0 to 6.5, with the exception of pullulanase type II from *Pyrodictium abyssi*, which exhibits its highest activity at pH 9.0 **(Bertoldo and Antranikian. 2002).** In accordance with the acidic catalysis mechanism proposed for amylolytic enzymes (**Kim *et al*., 1999),** the recombinant TK-PUL has an optimum pH of 3.5 in sodium acetate and 4.2 in sodium citrate buffer and is active over a broad pH range (3.0 to 8.5). α-amylase purified from *B*. *cereus* GUF8 appeared as a single polypeptide with a molecular weight of about 56 kDa similar to *B*. *licheniformis* and *B*. *amyloliquefaciens* α-amylases. The enzyme activity was measured in presence of various ions at a final concentration of 1 mM in the reaction solution. Of all the metal ions tested, Ag^{+}, Urea and Zn^{2+} showed remarkable inhibitory effects because they reduced the enzyme activity by 64 %, 42 % and 12 % respectively (Fig.21) but other metals as Co, EDTA and K^{+}showed stimulating effects of 34 %, 19.2% and 14%, respectively. The results are in close agreement with the study of recombinant expression of amylase from *Bacillus subtilis* in *E. coli* by (**Zafar ., *et al.* 2014**), that showed increase in the enzyme activity by Co but amylase activity was inhibited by Zn^{2+}, Hg^{2+} and Ni^{2+} ions. Although it is difficult to compare the kinetic values of α-amylases reported by other groups in view of the different starch substrates and their assay conditions, the *Km* value of *B*. *cereus* GUF8 α-amylase, 1.4 mg/ml, is within the range of majority of other α-amylases (0.35 to 4.7 mg/ml) (**Aguilar *et al*., 2000**). The hydrolysis products of soluble starch were determined by TLC analysis. The major end products of the enzyme were G2-G5 together with smaller amounts of higher maltooligosaccharides, however, it was not able to produce glucose, These indicate that the amylolytic enzyme from *B*. *cereus* GUF8 hydrolyzes starch by randomly cleaving internal α-1,4-glycosidic linkages and therefore the reaction proceeds in an endo-type fashion (**Mezghani and Bejar. 1999; Prieto *et al.,* 1995**).Industrial starch hydrolysis is a two-step process, amylase and pullulanase is normally added during the second step (saccharification) to hydrolyze α -1,6 linkages at branch points that were not attacked by α -amylase in the first step (liquefaction) (**Lévêque *et al.* 2000)**. Complete solubilization of starch in water can only be achieved at a temperature above 100°C, and the natural pH of this solution is 4.5. Therefore, the use of highly thermostable amylolytic enzymes capable of working efficiently in this acidic environment would directly benefit the starch industry **(Van der Maarel, 2002.).** We describe here alkaliphilic amylase -hydrolyzing enzyme from *Bacillus. licheniformus* The amino acid sequence of TK-PUL displayed homology with type II pullulanases, and it was annotated accordingly during the analysis of the gene sequence of *Bacillus. licheniformus* **(Fukui *et al* ., 2005).** A major problem for the starch industry is that Ca^{+2} has to be added to starch slurry to enhance the thermostability and activity of amylases. This added Ca^{+2} inhibits the activity of glucose isomerase, which is used in later steps for isomerization of glucose to fructose during high-fructose syrup production **(Wang *et al*., 2007).** Calcium oxalate is also produced as a waste product and deposits in the pipes and heat exchangers. This deposition chokes the pipes and in turn increases the production cost. With

the development of Ca^{+2}independent thermostable enzymes, this problem can be solved. AmyA does not need $Ca^{+2}$for activity and thermostability. Its Ca^{+2} independence, along with its high thermostability and pH optima of 9.0 in buffer, make AmyA an attractive candidate for application in the starch industry.As both the α -1,4- and α -1,6-hydrolyzing activities of recombinant Amylase A was inhibited at the same rate in the presence of 0.1% cyclodextrin, 0.01% *p*-chloromercuribenzoic acid, and 0.01% *N*-bromosuccinimide, it can be speculated that recombinant AmyA possesses a single active site for the hydrolysis of both α -1,4 and α -1,6 glucosidic linkages. However, detailed studies are required to reach such a conclusion. Amylopullulanases from *D. mucosus* **(Duffner *et al.,* 2000.),** *Thermoanaerobium* strain Tok6-B1 **(Plant *et al.*, 1987),** and *C. thermohydrosulfuricum* **(Mathupala *et al.,* 1990)** have been shown to possess one active site that hydrolyzing both 1,4 linkage and 1,6 linkage , whereas amylopullulanasesN from *Bacillus circulans* F-2 **(Kim and Kim . 1995),** *Bacillus* sp. strain KSM- 1378 **(Hatada *et al*., 1996)** and *P. woesei* (**Rudiger *et al.,* 1995**) have been reported to possess two different active sites responsible for dual catalytic activities. The amylase activity was slightly inhibited in the presence, possibly due to substrate competition. The slight inhibition may be attributed to a possible catalytic site being involved in both types of reactions. Among previously reported amylases, only two enzymes, from *T. aggregans* **(Niehaus *et al*., 2000)** and *D. mucosus* **(MacGregor *et al.,* 2001),** were able to hydrolyze cyclodextrins, while others were competitively inhibited. AmyA was able to hydrolyze Amylose into glucose. These annotations were based on the amino acid sequence comparisons. Sequence-based classification of putative enzymes may not always be correct, as observed in the case of the well-characterized branching enzyme TK1436 from *T. kodakarensis*. TK1436 was annotated as a "probable α -amylase" in the genome sequence, but the biochemical characterization was in contrast with the sequence-based annotation and showed that it was a branching enzyme **(Murakami *et al*., 2006)**.

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