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## Optimization of Xylanase and $\alpha$ -Amylase Production by Alkaline and Thermophilic *Bacillus* Isolate KH-13

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### Abstract

Alkaline, and thermophilic *Bacillus* sp. strain KH-13, which produces  $\alpha$ -amylase and xylanase was isolated from Caspian Sea in Iran. Characterization of strain KH-13 by 16S rRNA gene analysis found it closest member of *Bacillus* sp. M26 with a similarity of 98%. This bacterial isolate was mutagenized by treatment with Ethidium bromide (EtBr) and 4 mutant variants were obtained. Wild type strain KH-13 and its mutant variants KH13-M3, and KH13-M4 were selected for partial characterization. Maximum  $\alpha$ -amylase productions were achieved at the end of 12 h of growth for KH-13 and KH13-M3. Besides, maximum xylanase productions were achieved at the end of 24 and 48 h of growth for KH-13 and KH13-M4, respectively. The optimum temperature values of the KH-13 and KH13-M3  $\alpha$ -amylases were found to be 40 and 50 °C, respectively. An optimum pH value of the both enzymes was 6.5. The optimum temperature values of the KH-13 and KH13-M4 xylanases were found to be 50 and 60 °C, respectively. An optimum pH value of the both enzymes was 9.5.

**Keywords:** Alkaline, *Bacillus*, characterization, Ethidium bromide, isolation, mutagenesis, thermophile, xylanase,  $\alpha$ -amylase

### 1. Introduction

The use of microbial enzymes in industrial areas has been increasing because of its economical production and immobilization of unsolvable materials in water and durable use in respect to biotechnological activities [1]. Among the microorganisms, *Bacillus* species are good secretors of extracellular enzymes such as amylase, arabinase, cellulase, lipase, protease, and xylanase which play important roles in many biotechnological processes [2]. For applications in industrial processes, the enzymes should be stable at high temperature, pH, presence of salts, solvents, toxicants etc. [3]. Microbial hemicellulases, especially xylanases, have important applications in industry due to their enormous potential to modify and transform the lignocellulose and cell wall materials abundant in vegetal biomass which is used in a wide variety of industrial processes. Xylanases are used as additives in animal feeds for monogastric animals, together with cellulases, pectinases and many other depolymerizing enzymes. Enzyme degradation of arabinoxylans, commonly found as ingredients of feeds, reduces the viscosity of the raw materials thus facilitating better mobility and absorption of other components of the feed and improving nutritional value [4]. The incorporation of xylanase into the rye- or wheat-based diets of broiler chickens resulted in an improvement in weight of chicks and their feed conversion efficiency [5]. Similar improvements can be obtained for pigs fed on a wheat-based diet supplemented with xylanases and phospholipases [6].  $\alpha$ -Amylases (endo-1,4- $\alpha$ -D-glucan glucanohydrolase, EC 3.2.1.1) are one of the most important groups of industrial enzymes which hydrolyze starch molecules to give diverse products including dextrans and progressively smaller polymers composed of glucose units [7,8]. They find applications in the sugar, baking, brewing, paper, textile, distilling industries [9]. The main advantages of using microorganisms for production of amylases are their huge economical production capacity and their easy manipulation to obtain enzymes of desired characteristics [10].

$\alpha$ -Amylases have been developed from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have important applications in industrial areas [11]. Nevertheless, bacterial  $\alpha$ -amylases particularly *Bacillus* amylases are more desirable compared to fungal  $\alpha$ -amylases because of their heat stability [12]. The most plentiful used

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bacterial  $\alpha$ -amylases were derived from *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothersophilus* [13].

Most of the strains used for enzyme production have been improved cloning or mutation methods. UV and chemicals such as ethyl methyl sulfonate (EMS), nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and Ethidium bromide (EtBr) were found to be suitable mutagens for the improvement of enzyme production by microorganisms and obtained mutants have a higher capacity for amylase and xylanase productions [14,15,16,17]. The present study was aimed at isolation, identification of *Bacillus* sp. strain with  $\alpha$ -amylase and xylanase activity and improvement of the enzyme production with mutation by Ethidium bromide.

## 2. Materials and Methods

### Microbial cultures

*Bacillus* sp. KH-13 was isolated from coast sediment samples collected from Caspian Sea, Iran. To select the Gram-positive spore-forming bacteria *Bacillus* sp., soil samples were incubated at 80 °C for 10 min [18]. The isolates were cultivated in Luria Bertani (LB) medium containing (g L<sup>-1</sup>) tryptone 10, yeast extract 5, NaCl 10 (pH 9.0) for 24 h at 55 °C with shaking at 200 rpm. The isolates were screened for  $\alpha$ -amylase activity on LB-agar-starch plates containing (g L<sup>-1</sup>) tryptone 10, yeast extract 5, NaCl 10, starch 5, agar 15 (pH 9.0) [19], and for xylanase activity on same medium containing (g L<sup>-1</sup>) xylan 1, instead of starch at 55 °C. To detect  $\alpha$ -amylase activity, the starch containing plates were stained with iodine [20]. Xylanolytic isolates were selected by flooding the agar plates with Congo-red solution (0.1%) [21].

### 2.2. Molecular characterization

To determine the 16S rRNA sequences, the strain's genomic DNA was extracted as described previously [22]. To confirm the identity of the isolate, PCR amplification and sequencing of the 16S rRNA gene was done. The 16S rRNA gene was PCR-amplified from the genomic DNA using the bacterial universal primer set of 518F: (5'-CCAGCAGCCGCGTAATACG-3') and 800R: (5'-TACCAGGGTATCTAATCC-3'), which were also used for sequencing [23, 24]. The PCR reaction mixture included of 5  $\mu$ L of 10X PCR reaction buffer, 1  $\mu$ L of 40 mM dNTP mix (200  $\mu$ M each final), 1  $\mu$ L each of forward and reverse primers (20 pmol each primer), 0.5  $\mu$ L of Taq DNA polymerase (5 U/ $\mu$ L), and 3  $\mu$ L of genomic DNA template in a total volume of 25.0  $\mu$ L. The following amplification program was used: initial denaturation step at 95 °C for 15 min, which was followed by 30 cycles of denaturation at 95 °C for 35 sec, primer annealing at 55 °C for 35 sec, extension at 72 °C for 35 sec. A final extension step was performed at 72 °C for 5 min. The amplified PCR products were analyzed by 2% (wt/v) agarose gel. The sequence of 16S rRNA (1468 bp) was aligned by using the BLAST program to identify the most similar sequence in the database [25]. 16S rDNA sequences of different strains of *B. subtilis* and its phylogenetically related species and genera were downloaded from GenBank database (<http://www.ncbi.nlm.nih.gov/entrez>) and aligned to construct a neighbor-joining phylogenetic tree using Clustal W algorithm with the help of MEGA software version 4.1 [26].

### 2.3. Mutation procedures

The mutation procedures were performed as previously described [27]. Day 1: The overnight cultured isolate was diluted by sterile deionized water and then spread on LB-agar plates by glassy stick. The Ethidium bromide solution (20

mg/ml) was dropped to the pre-marked points on the plate with a micro-pipette. The surfaces of plates were dried for 15-20 minutes and then incubated for overnight at 55 °C. Day 2: The colonies around the toxic zones (Ethidium bromide dropped points) were picked onto new LB-agar plates containing soluble starch (0.5% wt/v) and xylan separately with sterile toothpicks. The plates were incubated at 55 °C for 24 h. Day 3: The plates were screened and selected for  $\alpha$ -amylase and xylanase activities according to zone diameters. All mutant variants were stored in sterile glycerol (10% v/v) at -20 °C for further studies.

### 2.4. Enzyme production

The wild type and mutant variants were grown up in LB medium at 55 °C with shaking at 200 rpm for 24 h. After removal of cells by centrifugation (Hettich Universal EBA12) (5,000 rpm, 10 min), the supernatant was used for enzyme assay.

### 2.5. Enzyme assay

$\alpha$ -Amylases were assayed by adding 1 mL enzyme to 1 mL soluble starch (2% wt/v) in 50 mM tris buffer (pH 9.0) and incubated at 55 °C for 30 min. The reaction was stopped by adding 3 ml of 3, 5-dinitrosalicylic acid reagent and  $A_{540\text{ nm}}$  was measured in a Pharmacia spectrophotometer [28]. One enzyme unit is defined as the amount of enzyme releasing 1 mmol of glucose from the substrate in 1 min at 55 °C. Xylanases were assayed in the same way using 1 mL xylan (2% wt/v) as substrate instead of starch.

### 2.6. Protein determination

Proteins of wild type and mutant variants were estimated as described by Lowry et al., [29] using bovine serum albumin as the standard.

### 2.7. Effect of incubation period

The effect of incubation period was determined by assaying the enzyme activity in different incubation periods (12, 24, 36, 48, 60, and 72 h) [28].

### 2.8. Effect of pH and temperature on enzyme activity

Temperature and pH effects on enzyme activities were assayed at different temperatures ranging from 30 to 100 °C and at pH values ranging from 6 to 11 for 30 min [28]. Following buffers were used in the reactions: 50 mM Na-phosphate (pH 6-7) and 50 mM Tris (pH 7-11) [30].

### 2.9. Statistical analyses

The tests were performed in four replications. Standard error was calculated using the statistical software SPSS version 19.0. All analyses were performed at  $p \leq 0.05$ .

### 2.10. SDS-PAGE and zymogram analysis

SDS-PAGE and SDS-Starch-PAGE (0.2% wt/v starch) were done as described by Laemmli [31] with slab gels (12% wt/v acrylamide). After the electrophoresis, the gel was stained for 1 hour with Coomassie blue R 250 dye in methanol-acetic acid-water solution (4:1:5, by volume) and destained in the same solution without dye [30]. For zymogram of amylase activity by SDS-Starch-PAGE, SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2), isopropanol 40% v/v for 1 h and 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2) for 1 h, respectively. Renaturation of enzyme was carried out by keeping the gel overnight in a solution containing 50 mM

Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4 °C. Gel was then transferred onto a glass plate, sealed with film, and incubated at 55 °C for 4 h. Gel was stained in a solution of iodine containing (g L<sup>-1</sup>) iodine 5, KI 50, for 30 min, clear band indicating the presence of amylase activity [30-32]. The molecular mass of the enzyme was finally estimated from the position of standard proteins.

### 3. Results

The alkaline, and thermophilic strain *Bacillus* sp. KH-13 producing extracellular α-amylase and xylanase was isolated

from Caspian Sea located in Iran. The bacterium was Gram positive, rod shaped, spore forming and aerobic. The optimal growth was observed between pH 6.0-8.5 for α-amylase (with optimal pH of 7.0) and 9.0-10.0 for xylanase (with optimal pH of 9.5) at 55 °C.

#### 3.1. Identification of the strain and 16S rRNA analysis

The isolate KH-13 was identified according to the 16S ribosomal RNA genetic analysis. BLAST analysis of the strain revealed that it had a closest match (98%) with *Bacillus* sp. M26 (Figure 1. and Figure 2.).

KH-13	9	ATCATTGGCGGT-AAGCGCGCGCAGCGGT-CCFTT-AGTCTGATGTGAAATCTTGGCGC	65
Ref	512	ATCATTGGCGGTAAAGCGCGCGCAGCGGTCCCTTAAAGTCTGATGTGAAATCTTGGCGC	571
KH-13	66	TCAACCGCTAAGCGGTCATTGGAA-CTGGGGACTTGAGTGCAGGAGAGAAAGCGGAAT	124
Ref	572	TCAACCG-TAAGCGGTCATTGGAACTGGGGACTTGAGTGCAGGAGAGAAAGCGGAAT	630
KH-13	125	TCCATGTGTAGCGGTGAAATCGGTAGAGATATGGAGGAACACCACTGGCGAAGCGGCTT	184
Ref	631	TCCATGTGTAGCGGTGAAATCGGTAGAGATATGGAGGAACACCACTGGCGAAGCGGCTT	690
KH-13	185	TCTGCCTGTAACCTGACGCTGAGCGCGAAAGCGTGGGAGCAACAGGATTAGATACCC	244
Ref	691	TCTGCCTGTAACCTGACGCTGAGCGCGAAAGCGTGGGAGCAACAGGATTAGATACCC	750
KH-13	245	TGGT	248
Ref	751	TGGT	754

Fig 1: Comparison of the 16S rRNA genes from *Bacillus* sp. KH-13 and *Bacillus* sp. M26 strain



Fig 2: Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of *Bacillus* sp. KH-13

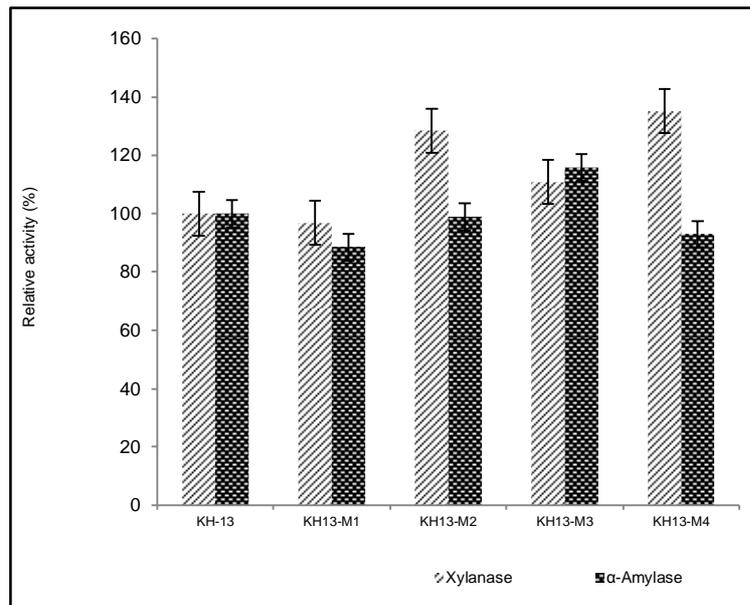
### 3.2. The mutation tests on the KH-13 isolate

After processing of *Bacillus* sp. KH-13 by EtBr, four mutant variants (KH13-M1, KH13-M2, KH13-M3, and KH13-M4) were obtained. According to diameters of amylase and xylanase zones on LB-agar plates, mutant variants were compared with wild type strain. With these results, mutant variant KH13-M3 and KH13-M4 were selected for further enzymatic analysis.

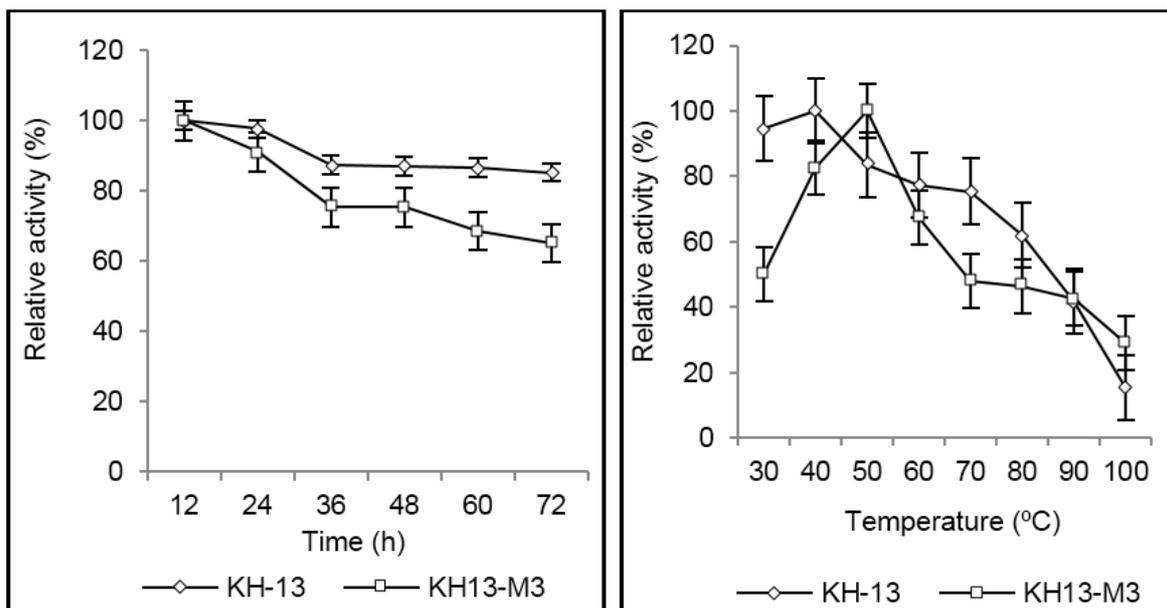
### 3.3. Enzyme properties

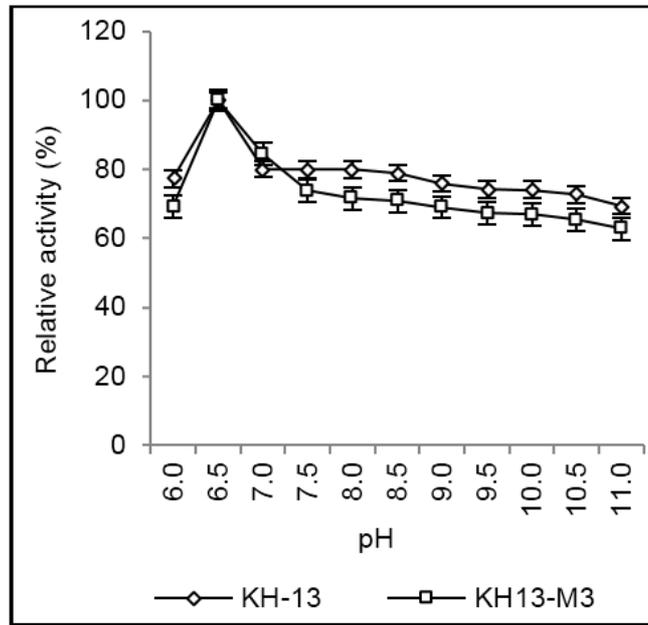
Specific  $\alpha$ -amylase activity of *Bacillus* sp. KH-13 was 4.38 U mL<sup>-1</sup> min<sup>-1</sup>. After treatment by EtBr, specific enzyme activities were increased to 4.76, 5.35, and 5.05 U mL<sup>-1</sup> min<sup>-1</sup> for mutant variants KH13-M1, KH13-M3 and KH13-M4, respectively. But specific enzyme activity of KH13-M2 was same as

obtained with wild type  $\alpha$ -amylase with the value of 4.37 U mL<sup>-1</sup> min<sup>-1</sup>. On the other hand,  $\alpha$ -amylase production decreased in mutant variants KH13-M1, KH13-M2 and KH13-M4 down to 11.47, 1.08, and 7.04%, respectively. On the contrary,  $\alpha$ -amylase production of mutant variant KH13-M3 increased up to 15.74% (Figure 3.). Specific xylanase activity was increased to 2.45, 2.12 and 2.58 U mL<sup>-1</sup> min<sup>-1</sup> for mutant variants KH13-M2, KH13-M3 and KH13-M4, respectively from 1.9 U mL<sup>-1</sup> min<sup>-1</sup> for wild type strain KH-13. Specific enzyme activity of KH13-M1 was found to be 1.85 U mL<sup>-1</sup> min<sup>-1</sup>. On the other hand, xylanase production increased in mutant variants KH13-M2, KH13-M3 and KH13-M4 up to 28.42, 10.87 and 35.08%, respectively. On the contrary, xylanase production of mutant variant KH13-M1 decreased down to 3.16% (Figure 3.).

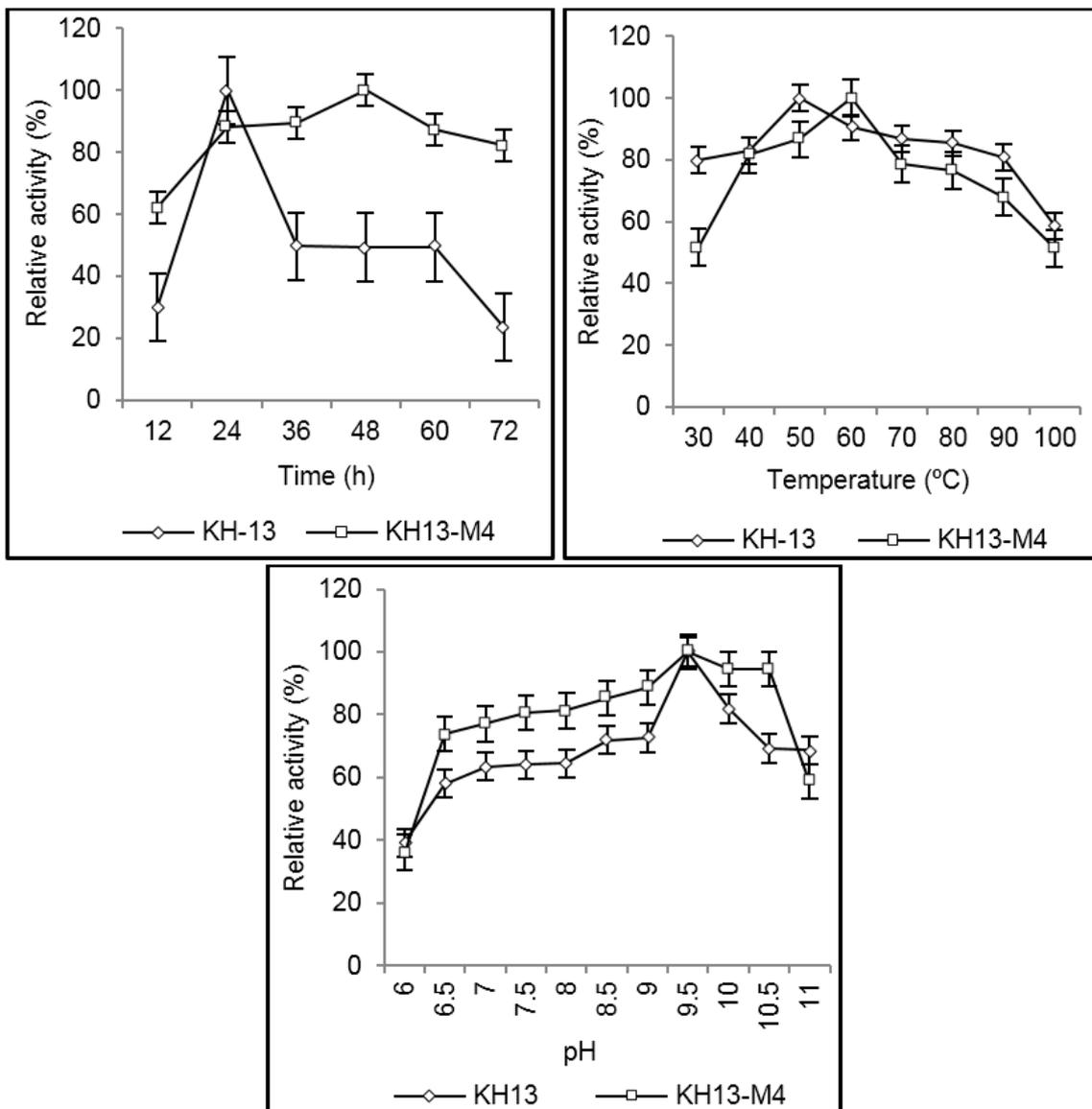


**Fig 3:** Comparison of native  $\alpha$ -amylase and xylanase with the mutant variants





**Fig 4:** Enzymatic properties of native KH-13 and mutant KH13-M3 variant  $\alpha$ -amylases (A: Enzyme production depending on time intervals, B: The effect of temperature on  $\alpha$ -amylases, C: The effect of pH on  $\alpha$ -amylases)



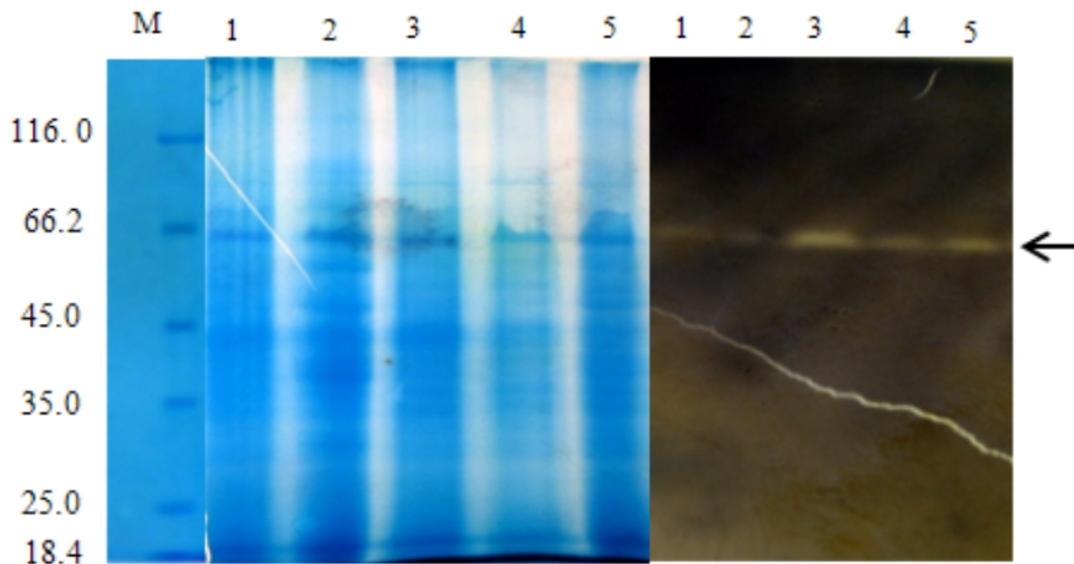
**Fig 5:** Enzymatic properties of native KH-13 and mutant KH13-M4 variant xylanases (A: Enzyme production depending on time intervals, B: The effect of temperature on xylanases, C: The effect of pH on xylanases)

Production of  $\alpha$ -amylases and xylanases at different time intervals as investigated. Maximum  $\alpha$ -amylase production was recorded after 12 h at 55 °C for KH-13 and KH13-M3 variants (Figure 4.A). Nevertheless, maximum xylanase production was recorded after 24 and 48 h at 55 °C for KH-13 and KH13-M4, respectively (Figure 5.A). The optimum  $\alpha$ -amylase activities were observed at 40 and 50 °C for KH-13 and KH13-M3, respectively (Figure 4. B). An optimum pH value of both the enzymes was 6.5 (Figure 4.C). The enzyme activities were decreased after incubation for 30 min at 60 °C. The optimum xylanase activities were observed at 50 and 60 °C for KH-13 and KH13-M4, respectively (Figure 5.B). An

optimum pH value of both the xylanases was 9.5 (Fig.-5C). Both  $\alpha$ -amylases and xylanases showed similar graphical properties for pH and temperature values. Similarly, both  $\alpha$ -amylase and xylanase activities were decreased after incubation for 30 min at 60°C.

### 3.4. Determination of molecular weight

Molecular weights of wild type and mutant  $\alpha$ -amylases determined by SDS-Starch-PAGE electrophoresis revealed single band showing  $\alpha$ -amylase activity in gel using BioCapt MW software. The molecular mass of bands was 66 kDa (Figure 6.).



**Fig 6:** Zymogram analysis of  $\alpha$ -amylases on SDS-PAGE. The gel was cut into two pieces, the marker and total proteins were visualized with Coomassie brilliant blue staining and the activity of enzyme revealed by iodine (M: Marker, 1: Wild type strain KH-13 protein samples, 2-5: Mutant variants protein samples from KH-13-M1 to KH-13-M4, respectively).

## 4. Discussion

In the present study, soil samples were collected from Caspian Sea of Iran and used for isolation of Gram (+), spore forming, and aerobic bacterial strains. About 220 strains were isolated and screened for  $\alpha$ -amylase and xylanase activity. Among these isolates, 9 bacteria showed both  $\alpha$ -amylase and xylanase activity on LB-agar plate containing starch and xylan. Among these isolates, the *Bacillus* sp. KH-13 was selected for mutation and further studies because of its maximum  $\alpha$ -amylase and xylanase activities according to hollow zones around the colony.  $\alpha$ -Amylases from alkaline and thermophilic *Bacillus* species have been reported previously [33, 34, 35, 36, 37]. Most of the *Bacillus* strains used commercially for the production of  $\alpha$ -amylases have an optimum pH between 6.0 and 9.0 for growth and enzyme production [30, 38]. Also xylanase from alkaline and thermophilic *Bacillus* species have been reported previously [37, 39, 40, 41, 42, 43]. Most of the *Bacillus* strains used commercially for the production of xylanase have an optimum pH between 6.0 and 9.0 for growth and enzyme production [44, 45, 46, 47, 48, 49]. The strain *Bacillus* sp. KH-13 was improved for  $\alpha$ -amylase and xylanase production. The chemical mutagen Ethidium bromide (EtBr) was used for mutation of the bacterial strain. The mutant *Bacillus* sp. KH13-M3 gave 5.35 U mL<sup>-1</sup> min<sup>-1</sup>  $\alpha$ -amylase which was around 1.22 fold higher than the parental strain. Several researchers have employed mutagenesis for  $\alpha$ -amylase production by exposing the cultures with UV or chemicals like Ethyl methanesulphonate (EMS), nitrous acid and Ethidium bromide

(EtBr) [50, 51, 52]. The mutant *Bacillus* sp. KH13-M4 gave 2.58 U mL<sup>-1</sup> min<sup>-1</sup> xylanase which was around 1.36 fold higher than the parental strain. The optimum incubation periods for our native enzyme and its mutant variant were 24 and 48 h, respectively. Besides, the native enzyme showed approximately 50% activity in the range of 36-60 h. The mutant variant KH13-M4 showed 76% activity after 24 h incubation, respectively. Since these incubation periods are shorter than those of bacteria and fungi, they are more acceptable for more economical production. The optimum pH value for native and mutant  $\alpha$ -amylases was 6.5. Both enzymes were active at slightly acidic and alkaline pH, with a range of pH activity (pH 6.0-11.0). The optimal temperature values for  $\alpha$ -amylase activity were 40 and 50°C for native and mutant enzymes, respectively. These pH and temperature values are similar to *Bacillus licheniformis* and *Gracilibacillus* [53], *Bacillus* sp. GUF8 [54], *Halomonas* sp. AAD21 [9], *Bacillus cereus* MS6 [55] enzymes. When the enzymatic properties were compared, it was clear that the temperature profile of our mutant enzyme was different from those of the known *Bacillus*  $\alpha$ -amylases. The optimal temperatures of most bacterial  $\alpha$ -amylases, including those from *Bacillus* sp. KH-13 are in the range of 40-90 °C. The activity of mutant enzyme was found to increase significantly at 50 °C in comparison with the native enzyme. It was reported that the broad range of temperatures and the enzyme's high activity at moderate and lower temperature values make enzymes highly attractive for both basic research studies and industrial processes [54]. The

molecular weight of  $\alpha$ -amylase was 66 kDa on SDS-PAGE. Similar findings between 55 kDa and 75 kDa have been reported earlier [37, 56, 57, 58, 59, 60]. These differences of molecular weights of  $\alpha$ -amylases depend on the genes from the organisms. Members of the genus *Bacillus* produce extracellular xylanases which are industrially important. Our thermophilic and alkaline tolerant isolate *Bacillus* sp. KH-13 also produces xylanase. The optimum pH for KH-13 and its mutant variant KH13-M4 xylanases was 9.5. However, the pH optimum for many bacterial xylanases was mainly reported in the neutral pH range. For example, xylanases isolated from *Bacillus* sp. [61], *Bacillus* sp. DM-15 [42], *B. licheniformis* [62] exhibited optimum pH of 6.5. Only a few xylanases isolated from bacteria, such as, *B. subtilis* have an optimum pH of 9.0 [63]. The xylanase from *Bacillus* sp KH-13 was thermostable up to 90 °C with optimum temperature of 50 °C. Mutation increased the optimum temperature up to 60 °C. The wild type and mutant enzymes retained their 81 and 68% activities at 90 °C. Similar results were also obtained for xylanase of *B. subtilis* [63]. Sriprang et al [64] have improved the optimum temperature of native fungal xylanase from 50 °C up to 55 and 60 °C for ST4 and ST5 mutant variants, respectively. The optimum pH and temperature for the KH-13 xylanase indicate the possible industrial use of the enzyme. According to the 16S rDNA sequence, the strain KH-13 is similar to *Bacillus* sp. M26 strain with the ratio of 98%.

## 5. Conclusion

In this study we isolated thermostable  $\alpha$ -amylase and xylanase producing wild type *Bacillus* strain KH-13 from Caspian Sea soil samples. After mutation by EtBr, the obtained mutant variant KH13-M3 and KH13-M4 was found to be more potent for  $\alpha$ -amylase and xylanase production.

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