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# New method for amylase production using metagenomics applications

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**ABSTRACT**

The traditional method of isolating enzymes from pure cultures only allows for the studying of a subset of the total naturally occurring microbiota in microorganism-rich environmental samples. Conventional methods are known to be able to cultivate less than 1% of all microorganisms found in nature. With the aim of isolating new enzymes and creating a special library for the amylase enzyme that has special capabilities from previously transplanted organisms, the metagenome technology has been directed. Soil DNA was isolated and the pUC19 vector was used for a metagenomic library creation. The resulted libraries were tested for enzymatic hydrolysis, and 8 clones out of 177 recombinant *Escherichia coli* clones found to have amylase activity by the fast traditional method for amylase screening. The libraries were examined for enzymes capable of breaking down starch. The best clones were selected to produce amylase. Two clones gave the highest amylase activity using quantitative methods. The inserted gene into clones were sequenced, and turned out to be an amylolytic enzyme that is new generated by a novel gene. For characterization, the amylase gene (SNSY) was over expressed and purified. The best AmylSNSY enzyme activity was found at pH (8.0), temperature (55°C) and resistance to NaCl up to 3 M. This research provided a simple and reliable method for cloning recent amylase genes from ecological metagenomes. In the future, the metagenomics guide method may be useful in increasing the collection of novel amylase from the environment

***Keywords:*** *Amylase, Cloning, Metagenome, Expression, Characterization*

## Introduction

Enzymes are macromolecular biocatalysts in living cells that cause and speed up thousands of biochemical and metabolic reactions. Enzymes are complex biocatalysts that hurry up a biological system's metabolic reactions (Aleem *et al*., 2018). Microbial enzymes are gaining a lot of attention because of their chemical stability, low cost, viability, efficiency, and environmental sustainability (Singh *et al*., 2019).

Amylase enzymes are a wide category of hydrolase enzymes that account for 25% of the total enzyme souk. The glycosyl hydrolase family includes α-amylases 13 (Sindhu *et al*., 2017). Amylases story

for roughly 30% of the inclusive enzyme market, with proteases coming in second (Gbenga *et al*., 2017, Wu *et al*., 2018). Aerobes and anaerobes both yield this enzyme (Raul *et al*., 2014; Singh and Kumari, 2016). Amylases from various cots are extensively used in industry for vital profitable tenders such as bread and baking, starch liquefaction, pharmaceutical, detergent, clothing, paper, alcohol production, brewing, cloth and leather, washing, washing-up liquid arrangements, starch coatings of paints products, and removal of starch (Gbenga *et al*., 2017; Singh and Kumari, 2016, Kaur *et al*., 2019). Despite the fact that novel α -amylases can be bent and recognized from an assortment of foundations, counting bacteria, fungi, and yeasts (Fang *et al*., 2019, Liu *et al*., 2019), more realistic methods have been developed to achieve this aim.

Metagenome can categorize microbial societies that can't be cultured by other methods using a practical performance. This performance has also assisted in the sighting of antibiotics (Sindhu *et al*., 2017). The metagenomic method is a viable option (Prayogo *et al*., 2020) Metagenomics is a system for learning DNA extracted from ecological trials without culturing or isolating microbial populations. This method was also used to discovery new microbial harvests and to sample microbial assortment from unlike eco-friendly niches. Every environment's DNA reservoir was assembled (Alves *et al*., 2018). Metagenomics is a performance for analyzing genetic material mined directly from eco-friendly samples. Using broadcast skills based on sequence and function, metagenomic reveals information about microbial populations of uncultivable species in environmental niches. Identification of novel bacteria or gene clusters encoding pollutan-degrading enzymes are examples of applications (Datta *et al*., 2020). Different metagenomic styles are speak to decide vital questions related to taxonomic assortment, and the characters played by them (Functional metagenomics) (Almeida *et al*., 2019).

## Materials and Methods

### Sample collection and preparation

Soil samples were taken from various waste sites in Alexandria, Egypt, transported to the laboratory, placed in a cold room, and then analysed.

### Chromosomal DNA preparation

An updated method of unique kit for DNA extraction from soil was used to extract genomic DNA (QIAGEN).

### DNA restriction digestion

In a 20 µl reaction volume, chromosomal DNA was digested by restriction enzymes (5 units) in presence of the suitable buffer (2 µl). Restriction enzyme (*Bam HI* and *EcoRI*) digestion was carried out under the reaction conditions recommended by the manufacturers for each enzyme (Fermentas).

### DNA ligation

A ligation was performed in a 20 µl reaction volume with 2µl of T4 DNA ligase buffer and 1 unit of T4 DNA ligase enzyme, followed by the insertion of digested DNA and the addition of vector (4:1). The reaction was carried out overnight at 16 °C. The screening and expression vector was pUC 19.

### Competent cells preparation and transformation

According to Sambrook *et al.* (1989), *DH5*α competent *E. coli* cells were prepared. The competent cells were frozen aliquots that were allowed to thaw on ice. The tube was then filled with DNA (ligation mixture) and incubated on ice for 20 minutes. The tube was heat-shocked for 60 seconds at 42°C, then 800 µl LB medium was added and the tube was incubated at 37 °C with shaking for one hour. On selective LB plates containing X-Gal and IPTG, aliquots 200 µl were scattered.

### Screening and measurement of enzyme activity

Pure clones were screened for amylase activity in LB (Ampicillin and IPTG) supplemented with 0.5 percent soluble starch for qualitative estimation of amylase enzymes. After adding the indicator (iodine pellet), a transparent halo appears around the colonies, indicating a positive enzyme activity. Soluble starch was used to estimate enzyme activity quantitatively for positive clones. This was accomplished by inoculating 1ml suspension from freshly prepared slant (18h) of each checked clone into 50ml of output LB (Ampicillin and IPTG) medium dispensed in a 250 ml Erlenmeyer flask. Using a reciprocal shaker, the inoculated flasks were incubated at 37 °C for 24 hours (200 rpm). The cells were separated by centrifugation at 5000 rpm for 15 minutes, and the pel let was used to measure enzyme activity at 37 °C using soluble starch substrate.

**Preparation of plasmid-DNA from *E. coli***

For positive amylase clones, GEBRI kit mini-plasmid extraction was performed as follows: cells (1.5 ml) from overnight culture were centrifuged at 7000 rpm and suspended in 300 µl of solution I, lysed by adding 300 µl of solution II with mild shaking. To precipitate the protein and genomic DNA, Solution III (300 µl) was applied, followed by centrifugation at 13000 rpm for 10 minutes. The plasmid DNA-containing supernatant was precipitated with isopropanol, washed with 70% (v/v) ethanol, and then suspended in 30 µl of water.

**Enzyme colorimetric assay**

Pellets of positive clones were sonicated in 2ml phosphate buffer pH 7 then amylase activity was measured by colorimetric method at $\lambda_{660nm}$ (Abdel-Fattah *et al.*, 2013).

**Sequence similarity**

The BLAST software (www.ncbi.nlm.gov/blast) was used to compare the similarity of the received PCR product (M13 F: AGGCCCTGCACCTGAAG M13 R: TCAGCGCCTGGTACC, Ali & Soliman, 2019) with the previously submitted sequences in the database. In addition, the sequence retrieval system SwissProt http://hcuge.ch/srs5/ was used to compare several amino acids.

**Characterization of amylase**

**Determination of the optimum temperature**

In a 50 mM tris-HCl buffer pH 7, the temperature optimum of amylase was calculated over a temperature range of 30-80 °C.

**Determination of the optimum pH of the enzyme activity**

Over a pH spectrum, the optimum pH of amylase was calculated (3.5-10). Citric acid, phosphate and tris-HCl at 50 mM buffer intensity were used in this test. For 30 minutes, reaction mixtures were permitted to stand at 45 °C with shaking.

**Thermal stability**

Amylase's thermal stability was measured for up to 2 hours at temperatures ranging from 25 to 75 degrees Celsius. The enzyme's stability was measured at -20 degrees Celsius for up to two months without thawing. The stability of the sample was also tested by gradually weakly thawing it up to 10 days enzyme solution in 50 mM tris-HCl buffer pH 7 at different time intervals and determining the residual activity.

**pH stability of enzyme**

The pH stability of enzymewas tested for up to 10 weeks at pH 7, 7.5, 8, and 8.5 using the optimum temperature.

**Effect of different compounds on enzyme activity**

The crude enzyme was tested against a variety of cations (MgCl2, MgSO4, CaCl2, ZnCl2, NaCl, FeSO4, MnSO4, and CuSO4) at concentrations of 1 mM, a chelating agent (EDTA) at concentrations of 1 mM, a solvent (DMSO, methanol, ethanol, isopropanol, glycerol) at concentrations of 1 percent (v/v). The tested enzyme was handled separately with the tested agent for 15 minutes; the residual activity was measured and subtracted from the activity of the untreated enzyme to determine whether the tested compound inhibited or activated the enzyme.

## Results and Discussion

The aim of this study is to determine the frequency of positive amylase clones in soil from Wadi El Natrun, Egypt, using a metagenomic technique. During the year 2018, a screening analysis was carried out. With a concentration of 13389 ng/ µl, DNA purity was found to be (1.81) Following ligation, 177 clones were obtained from DNA digested and ligated to the same enzyme-digested pUC19 (78 clones from digested DNA using *BamHI* and 99 clones selected from DNA digested with *EcoRI*).

**Amylase-positive screening and selection**

Qualitative screening of amylase was performed on soluble starch agar plates at 37 °C in a screening programed for the isolation of amylase enzymes generating clones (Fig. 1). Only 2 clones showed a promising result in terms of distinctive behaviour among all tested 8 clones, which showed variance in their hydrolytic potential represented by zone diameter in mm. The quantitative estimation of amylase by selected clones revealed that both A and B clones gave (83 and 58 U/min/ml, respectively) comparing to vector free gene (C) (Fig. 1).

**Promising Clones' Molecular Identification**

The Nano drop method was used to estimate the

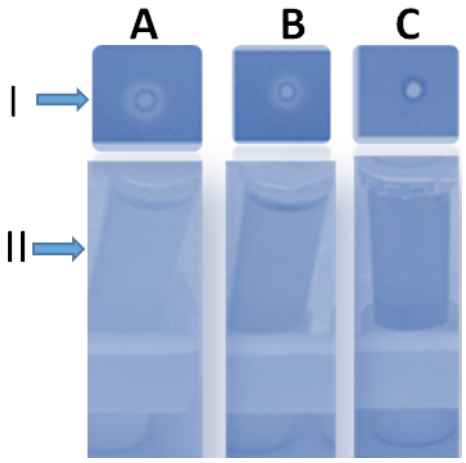


**Fig. 1.** Activity verification for active recombinant amylase clones (A & B). I: Well-cut method and II calorimetric method.

concentration and purification of Plasmid extracted from the selected amylase clones (SNSY A and B). Result in showed that the plasmid concentration was various among the clones (1876 and 1913 ng/µl) with purity (1.8 and 2). PCR was applied to the selected clones coded (A and B). Molecular identification of the Promising clone A (selected from *EcoRI*-digested DNA) was done with the M13 universal primer. A 1% agarose gel was used to examine the PCR products (Fig. 2).

Despite the high amylase performance of clone A (83 u/min/ml), the researchers opted to use the Gen Bank database to identify and classify the amylase gene. A PCR fragment (1350 bp) was amplified using M13 primer and plasmid extraction from clone A.One consensus partial sequence (1098bps) (Amyl SNSY clone A) was obtained after sequence assembly. On the SDS-page, the molecular weight of AmylSNSY was 50KD, as determined by zymogram. A partial amino acid sequence consists of 366 aa was compared to the data in NCBI Genbank via protein BLAST, where it showed 41% Identity to Crystal Structure of *Anoxybacillus* Alpha-amylase Provides Insights into a New Glycosyl Hydrolase Subclass (*Anoxybacillus ayderensis*)(Ac: 5A2B_A). Multiple sequence alignments were performed with default parameters for phylogenetic tree construction. A phylogenetic tree was estimated for each protein using MEGA (version 5.2) and the maximum-likelyhood approach based on the results of several sequence alignments (Fig. 2).

**Characterization of recombinant amylase**

**Effect of temperature**

**Optimum temperature**

Temperature plays a key role in confirming the protein's resistance to enzyme inhibition. If the temperature is raised above this point, the secondary, tertiary, and quaternary bonds in the enzyme will be broken, resulting in denaturation (Gomaa, 2013). Different temperatures (30-80 $^0$C) were tested to determine the optimal value of the enzyme, as described in materials and methods. For 30 minutes, the enzyme was incubated at various temperatures. Figure 3shows the data plotted in a graph, where it was discovered that the optimal temperature for AmylSNSY was 55 $^0$C. Our findings were reliable

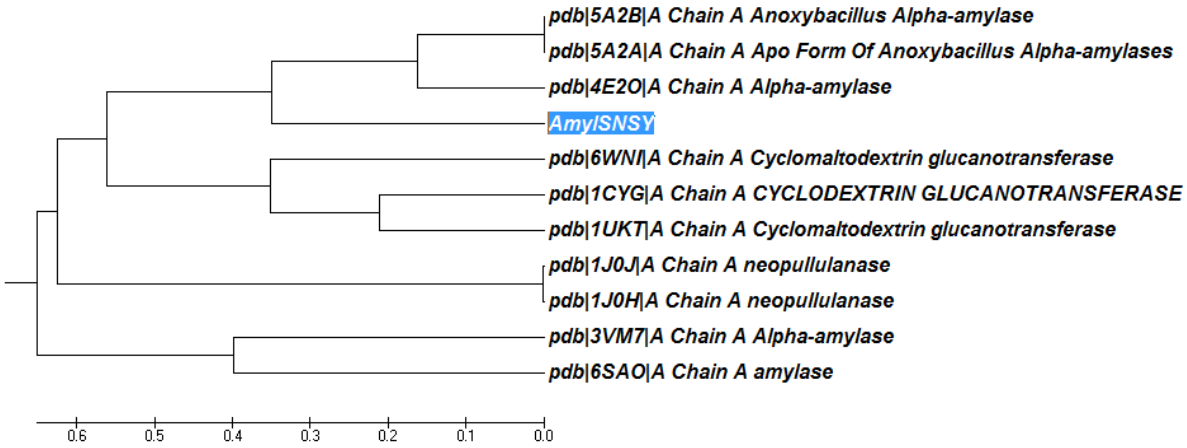


**Fig. 2.** Phylogenetic relation of AmylSNSY gene with amylase from the database. The dendogram was generated by the neighbour-joining method using MEGA 5 Software.

with those of Bhatt *et al.* (2020), who observed that amylase activity was maximum at 55 °C and dropped significantly below and above that temperature, and Yassin *et al.* (2021), who revealed that temperature had a generous impact on amylase output. The clones were cultivated and found to produce a lot of amylase at a high temperature range of 50-60 °C with maximum amylase production at 55 °C. Denaturation of proteins happened at temperatures above 55 °C, which may have impaired enzyme activity. *Bacillus cereus* and *Aspergillus oryzae* S2 Sahnoun *et al.* (2015) and Anto *et al.* (2006) reported similar findings. According to Shobana *et al.* (2017), the ideal temperature for the activity was found to be between 30 and 70 degrees Celsius. Kohli (2020) found that the purified enzyme was found stable at high-temperature range (40–80 °C), the enzyme activity was increased by increasing the temperature and showed optimal incubation temperature at 45 °C (32.7 U/ml) (Jana *et al.*, 2013).

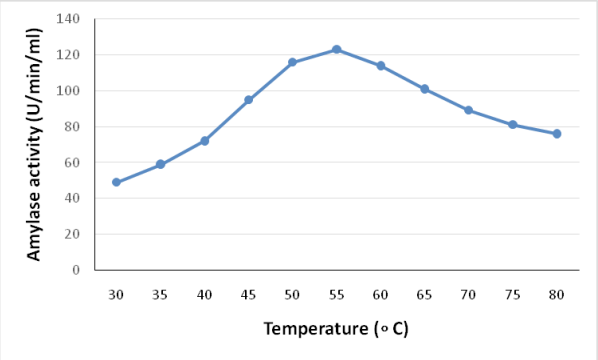


**Fig. 3.** Optimum temperature of recombinant amylase from clone AmylSNSY.

## Thermal stability

The residual activities were plotted against time exposure for each temperature 25-75 °C to determine the thermal stability of the recombinant AmylSNSY enzyme. Under the following reaction conditions (55 °C & pH 8 tris-HCl), residual activity was calculated. The amylase enzyme was fully stable at 25, 30, and 35 degrees Celsius for 5 hours, at 40 and 45 degrees Celsius for 90 minutes, and at 50 and 55 degrees Celsius for 60 minutes. Increasing the temperature to 60, 65, and 70 °C caused a notable decrease in thermal stability for recombinant AmylSNSY enzyme, while the enzyme losing 22, 29 and 39 percent after 15 minutes, respectively. The viability of recombinant Amyl SNSYenzyme was measured at -0°C without thawing for up to 10 weeks. The recombinant Amyl SNSYclone's enzyme was active in this state for 10 weeks without thawing, with a 6 percent loss in function. The stability of the enzyme was also tested by gradually weakly thawing the sample up to 10 times freezing and thawing for 20 days, and a loss of 83 percent was observed as presented in Fig. 4.

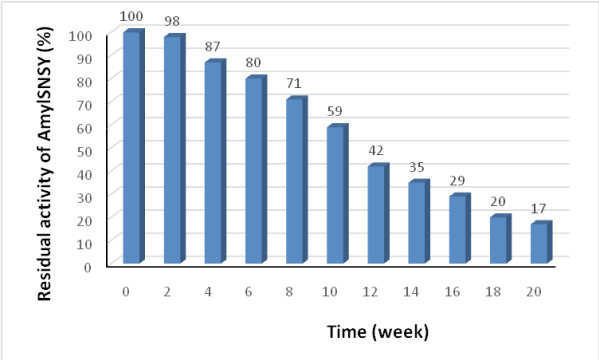


**Fig. 4.** Effect of thawing on amylase activity by AmylSNSY.

## Effect of pH (Optimum pH and pH stability)

The activity rate of the recombinant AmylSNSY enzyme was investigated as a function of pH from 3.5 to 10. This was done at 50 degrees Celsius with the following reaction conditions: citrate buffer for pH 3.5-5, phosphate buffer for pH 6-7, and tris-HCl for pH 7.5-10. It was discovered that the enzyme prefers to function in an alkaline environment, using a pH of 8 being the maximum (Fig. 5). The enzyme stability was measured in an alkaline environment (pH 7.5-8.5) for 6 hours at room temperature, and it demonstrated full pH stability at all of the pHs tested. The viability of the recombinant AmylSNSY enzymes was also tested after 10 weeks of storage at various pHs (7.5, 8, and 8.5) and cooling (-20 °C) as showed in Fig. 6. The enzymes measured were stable, with a small lack of activity at pH 7.5. The current findings corroborated with Mamo and Gessesse (1997), who discovered that *Bacillus* sp. WN11 alpha amylase was active at pH 5.5 and stable in the pH range of 5.5–9.0 (Bradford, 1976; Kucuk and Gulcin, 2016). Some amylases have pH values ranging from acidic to neutral. Amylase from *B. licheformis* MTC1483 has an optimal pH of 8.0, according to Kaur *et al.* (2019). On the other hand, the results are almost congruent to those found for amylases from *B. Amy175* had the highest activity at

pH 8.0 (Wang *et al*., 2018). It is, however, lower than the pH of amylase isolated from *B. subtilis* (pH 7) (Gbenga *et al*., 2017; Raul *et al*., 2014), *B. methylotrophicus* (pH 7) (Xie *et al*., 2014), *Bacillus* spp. from selected soil sample (pH 7) (Singh and Kumari 2016), *Bacillus* spp. (pH 9) (Tambekar *et al*., 2014), *B. subtilis*DM-03 (pH 9) (Das *et al*., 2004), *B. persicus* (pH 10) (Hadipour *et al*., 2016) and *Lysinibacillus xylanilyticus* (pH 10) (Tambekar *et al*., 2016). Amylase was most stable in the pH range of 4.5 to 8.0, and at pH 8.0, 75 percent of the activity was recovered which produced by *B. atrophaeus* NRC1 (Kaur *et al*., (2019). Nielsen and Borchert (2000) noted that in the starch industry, amylases must be active and have a stable profile behaviour at low pH, but in the detergent preparations sector, it must have stability at alkaline pH.

**Effect of different compounds on recombinant AmylSNSY enzyme activity**

Figure 7 showed the effect of different solvents on the residual amylase activity measured by percentage (%). Both of DMSO and glycerol at concentrations (1 and 10%) increased amylase activity to its maximal value of 100 percent. At a concentration of 1%, Ethanol, Isopropanol, Acetone, and Methanol experienced a minor decrease in the percentages of their enzyme residual activities (99,99,98 and 96%), whereas when used at a concentration of 10%, the residual activities reduced to (97,98%.93 and 96 respectively). DMSO significantly increased the enzyme's activity. Our findings were quite similar to those of (Wang *et al*., 2018), who discovered that DMSO had a substantial stimulatory effect on Amy175 cells.

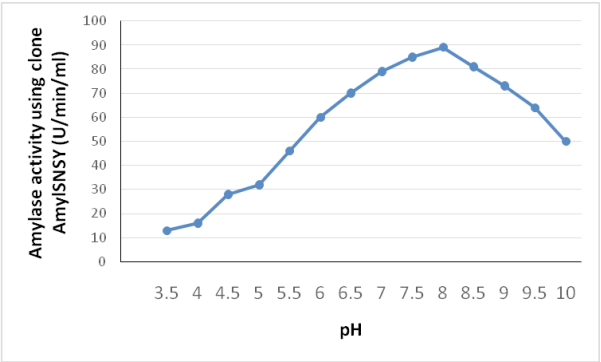


**Fig. 5.** Optimum pH of amylase using recombinant Amyl SNSY.

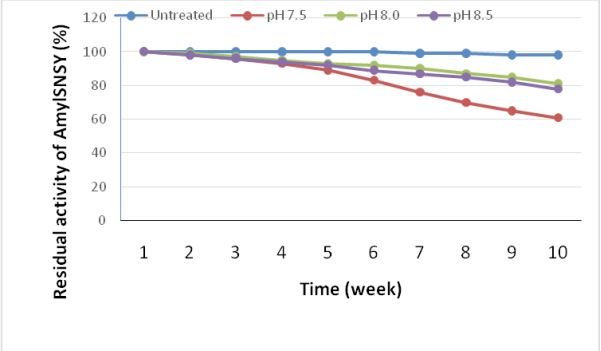


**Fig. 6.** Monitoring of pH stability for recombinant Amyl SNSY enzyme activity through long term preservation at -20 °C without thawing.

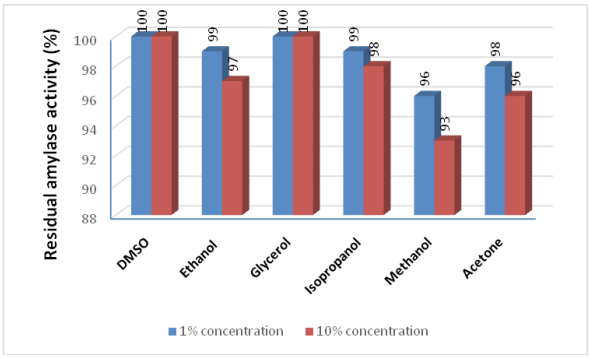


**Fig. 7.** Effect of different solvents (1 and 10% concentration) on recombinant amylSNSY enzyme activity

The effects of laundry detergent (Triton X-100, Tween-20, Tween-80, and SDS), chelating agents (EDTA), and salts ($CaCl_2$, NaCl) were shown in Figure 8. The crude AmylSNSY enzyme activity was assessed with (1 percent concentration). These findings were in agreement with those of Wang *et al.* (2018) and Adejuvon *et al.* (2018) who discovered that EDTA and Tween80 blocked amylase activity. In contrast, 1 mM sodium dodecyl sulphate (SDS) greatly activated amylase (Hmidet *et al*., 2009; Wang *et al*., 2018). Only around 26% of the enzyme's residual activity was decreased by EDTA. Our findings corroborate those of Hu and Liu (2021), who found that EDTA hindered significantly the activity to less than 50%. On the other hand, Ahmad *et al.* (2010) found that EDTA totally inhibited the enzyme activity of amylase from *Bacillus* species. The calcium ion is essential for the constancy of customary amylase used in the starch scarification business. In most of the cases, it was observed that $Ca^{2+}$ increases amylase activities and stability as those obtained by (Preseèki *et al*., (2013); Ghollasi *et al*., (2013) and Onodera *et al*., (2013). Many other authors have observed that the enzyme is $Ca^{2+}$ inde-

pendent (Xian *et al*., 2015) and that $Ca^{2+}$ even decreases the enzyme's activity and stability in some statuses (Han *et al*., 2013). The popular of amylases are metalloenzymes, which require $Ca^{2+}$ ions for activity, stability, and structural integrity. According to Gupta *et al.* (2003) this is due to the formation of a calcium sodium- calcium metal harmony in the main $Ca^{2+}$ binding site through A and B of the enzyme bridging domains. Therefore, the amylase of the present study seemed to act in a $Ca^{2+}$-dependent style. $Ca^{2+}$ also stimulated an amylase isolated from *Bacillus* sp. MRS6 (Sahoo *et al*., 2016). The amylase enzyme was found to be halo tolerant in the presence of sodium chloride, with maximum activity at a concentration of 1%. These findings are akin to those obtained by Yassin *et al.* (2021) and a few halophile amylases published in the literature Sahoo *et al* (2016) and exhibited extreme salt-resistance with the maximum activity at 1 M NaCl. Also the enzyme was stimulated by Na+ and $Ca^{2+}$ (*Adejuvon et al*., 2018).

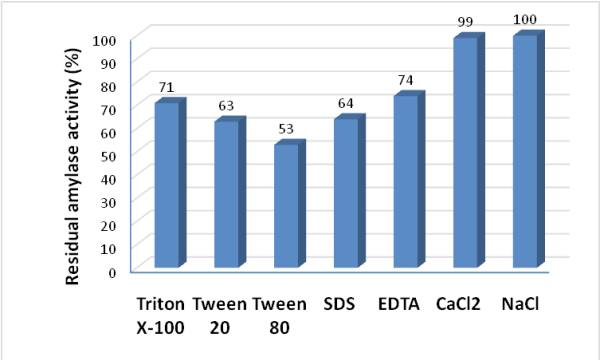


**Fig. 8.** Effect of detergents (Triton X-100, Tween-20, Tween-80 and SDS), chelating agents (EDTA) and salts ($CaCl_2$, NaCl) with (1% concentration) on recombinant AmylSNSY enzyme activity.

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