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# Optimization and immobilization of amylase produced by *Aspergillus terreus* using pomegranate peel waste

Nehad E. Ahmed, Aliaa R. El Shamy and Hassan M. Awad\* 

## Abstract

**Background:** Amylases are amongst the most important hydrolytic enzymes that are used in numerous industrial uses reaching for food to pharmaceuticals. Immobilization of enzymes can proposal several assistances as reusability and retrieval from their products improve strength under both operating and storing environments.

**Results:** Marine fungal isolate was recovered from red sea water at Sharm El-Sheikh Province and was tested for amylase activity using different agricultural wastes as substrate. It was found that pomegranate peel was the best substrate for amylase production (339 U/ml). Thus, it was subjected for identifying by 18S rDNA gene. The phylogenetic analysis results indicated that this fungal isolate belonged to *Aspergillus* species with similarity of 99% and named as *Aspergillus terrus* SS\_RS-NE. Its nucleotide sequences were deposited in NCBI GenBank under accession no. of MN901491. Some parameters affecting amylase activity using pomegranate peel as substrate were studied. The results denoted that, the highest amylase activity of 340.69 U/ml using 1.5% pomegranate peel at 30 °C, pH 6.0 on 5 days incubation time by *Aspergillus terreus*. The produced crude enzyme was partially purified with 80% ammonium sulfate followed by dialysis. The enzyme activity was 1246 U/ml and 2411 U/ml employing ammonium sulfate precipitation and dialysis respectively.

The partially purified amylase was immobilized with 2% sodium alginate and the results showed the highest immobilized enzyme yield was 92.8%. The characterizations of immobilized amylase were studied and the results indicated that, the maximal immobilized amylase activity was 2522.5 U/ml with 2% starch as a substrate at optimum pH value of 6.5, temperature at 60 °C and 10 min reaction time in comparison to maximal free amylase enzyme at pH 5, 50 °C after 40 min. The results also indicated the immobilized amylase was stable at 60 °C for 20 min.

**Conclusions:** *Aspergillus terrus* SS\_RS-NE (MN901491) was isolated and genetically identified. It has the ability to produce amylase enzyme using pomegranate peel waste with a yield of 339 U/ml. The crude enzyme was partially purified by ammonium sulfate followed by dialysis. The maximal immobilized amylase activity of 2522.5 U/ml was obtained under optimized some culture conditions and medium nutrient parameters.

**Keywords:** Marine fungal isolation and genotypic identification, Amylase production, Immobilized amylase, Pomegranate peel waste, Optimization production, Enzyme characterization and purification

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## Background

Amylases are the most important enzymes which hydrolyze starch molecules to dextrin and smaller polymers composed of glucose unit (Gupta et al. 2009). Amylases are a cluster of hydrolases that can definitely cleave the  $\alpha$ -glycosidic bond in starch. Amylases are classified as three important groups according to their action and properties to  $\alpha$ -amylase,  $\beta$ -amylase, and glucoamylase.  $\alpha$ -amylases (endo-1,4- $\alpha$ -D-glucan glucohydrolase, EC 3.2.1.1) are extracellular enzymes that catalyze hydrolysis of 1,4- $\alpha$ -D-glucosidic bonds in the internal of the starch molecule in a random way, creating branched and linear oligosaccharides (dextrin, maltose, maltotriose, glucose) of diverse chain length (Khan and Priya 2011).  $\beta$ -amylases (1,4- $\beta$ -D-glucan maltohydrolase, EC 3.2.1.2) are typically of plant source, but a rare microbial strains are similarly recognized to yield them. It is an exacting enzyme that splits non reducing ends of amylose, amylopectin, and glycogen molecule. Glucoamylase (Exo-1,4- $\alpha$ -D-glucan glucohydrolase, EC 3.1.2.3) hydrolysis sole glucose units from the non-reducing ends of amylose and amylopectin, these enzymes are the most important enzymes in biotechnology which constitute a class of industrial enzymes, approximately 25% of the world enzyme market. Amylases are the second largest group of enzymes used all over the world. They are widely used in biotechnology industries in starch saccharification processes, such as in textile industries, food and animal feed, detergents, fermented beverages and distilleries. In addition, they also have potential application in pharmaceutical, refined chemical, and bakery industries (Gurung et al. 2013). They can also be applied in recycling and paper production as well as in the juice industry, where they are used to clarify and decrease turbidity (Pandey et al. 2005).

Amylases are broadly dispersed in the environment and can be obtained from various sources, such as plants, animals, and microorganisms, but the main benefit of using microorganisms for the amylases production is the reasonable bulk production capability, also easily manipulated to obtain enzymes of desired characteristics (Karnwal and Nigam 2013). Amylase can be obtained from several fungi, yeast, bacteria and actinomycetes (El-Shahed et al. 2008). The fungal amylases are preferred over other microbial sources because of the accessibility and extraordinary efficiency of fungi, which are similarly agreeable to genomic guidance. Many fungi had been found to be good sources of amylolytic enzymes.

Amylase production has been reported in several different fungi species such as *Fusarium solani* (Kumar et al. 2012), *Aspergillus oryzae* (Ichinose et al. 2014), *Aspergillus niger* (Varalakshmi et al. 2009) and *Streptomyces erumpens* (Kar et al. 2008). Rizk et al. (2019) studied the production of  $\alpha$ -amylase by *Aspergillus niger*, they

showed that activity of  $\alpha$ -amylase increased by increasing pH value up to pH 6.5. The maximum activity of  $\alpha$ -amylase was recorded at temperature 45 °C. Rizk et al. (2019) mentioned that  $\alpha$ -amylase activity from *Aspergillus niger* increased by increasing pH value up to pH 6.5. The maximum  $\alpha$ -amylase activity was obtained at 45 °C. Also, Jain and Katyal (2018) optimized some production conditions of amylases from *Aspergillus* sp. and they indicated that the maximal amylase activity of 126.34 U/ml and 243.09 U/ml by *A. terreus* and *A. niger*, respectively.

Agro industrial remains are usually careful as the best substrate for the effective cost cutting in the production of alpha amylases. The choice of a substrate (agrarian waste) for enzyme production relies on a few factors for the most part correlated with the charge and accessibility of the substrate, the solid substrate not just delivers the nutrients to the microbial culture developing in it yet additionally fills in as a harbor for the cells (Nimkar et al. 2010). These agriculture wastelands comprise of C- and N-sources required for the growing and breakdown of microorganisms (Djekrif-Dakhmouche et al. 2006; Haq et al. 2005).

The utilization of enzymes in a free structure is uneconomic in light of the fact that the enzymes for the most part cannot be re-extracted at the end of the process; immobilized enzyme procedure forestalls enzyme misfortunes and simultaneously keeps up enzymes at extraordinary concentrations so as to diminish the expense of the enzymes (Baldino et al. 2001).

Overall, an immobilized form of amylase has exposed greater stability in comparing with free enzymes. It presented the reprocess of this biocatalyst for repetitive fermentation method and furthermore can be effortlessly isolated from the reaction mix (Konsoula and Liakopoulou-Kyriakides 2006). Among different immobilization strategies, capture is one of the most ideal strategies since it forestalls over the top loss of protein action, shields chemical from microbial infection (Cabral et al. 1993). Enzyme immobilization by capture creates the molecule structure which permits contact between the substrate and enzyme to be accomplished and, furthermore, it is conceivable to immobilize a numerous catalysts simultaneously (Hari et al. 1996). Physical ensnarement of  $\alpha$ -amylase in calcium alginate globules has appeared as a generally simple, quick, and safe strategy (Wolfgang Gerhartz 1990). de Souza et al. (2019) immobilized crude fungal amylase extracts from *Aspergillus* ATCC 3940 by diverse preparation techniques of alginate and chitosan. The maximal immobilization productivity achieved was 97.38% at pH 5 and 45 °C. The immobilized scheme might be enhanced by accumulated amylase concentration in immobilized

support, which would probable consequence in greater exchange rates of starch into sugars.

The objective of this study was isolation and molecular identification of fungal isolate, screening of different agricultural waste as substrates for maximal amylase production by *Aspergillus terreus*. Optimization of culture conditions, e.g., incubation time, temperature, and initial pH for amylase production and enzyme partially purified. Finally, enzyme immobilized using sodium alginate and studying its properties.

## Materials and methods

### Microorganism

The marine fungal strain was isolated from red sea water at Sharm El-Shiekh province and identified by 18S ribosomal RNA gene. The fungal culture was maintained on potato dextrose agar (PDA), incubated at 30 °C for 7 days and stored in the refrigerator at 4 °C.

### Molecular identification of fungal isolate

#### DNA isolation

DNA extraction was done by using the protocol of Gene Jet genomic DNA purification kit (Thermo# K0791) following the manufacturer's instruction.

#### PCR amplification and sequencing

The PCR amplification of 18S rDNA region was carried out following the manufacture of Maxima Hot Start PCR Master Mix (Mix (Thermo) #K0221). The 18SrDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify a 1500 bp fragment of the 18SrDNA region. The ITS1–5.8S–ITS2 genomic region was amplified from genomic DNA using the forward primer ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and the reverse primer ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White et al. (1990); Hamed et al. (2015)).

The PCR reaction was performed with 2 µl of 10× Taq PCR buffer, 1.6 µl from 2.5 mM dNTP Mixture, 1 µl of both forward and reverse primers (10 pmol/µl), 2 µl template genomic DNA (20 ng/µl), 0.2 µl from KOMA-Taq. (2.5 U/µl) and distilled water (HPLC grade) up to 20 µl. The reaction mixture as follows: initial denaturation at 95 °C for 1 min, 30 cycles denat. 95 °C for 30 s, annealing 55 °C for 2 min, extension 68 °C for 1.5 min, final extension 68 °C for 10 min for 1 cycle. After completion, the PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (10 mg/ml), to ensure that a fragment of the correct size had been amplified.

The amplification products were purified Montage PCR clean up kit (Millipore). The purified PCR products were sequenced by using the 2 primers that used before in the PCR reaction. Sequencing was performed by big dye terminator cycle Sequencing kit V.3.1 (Applied

Biosystems, USA). PCR products were resolved on an Applied Biosystems Model 3730XL Automated DNA sequencing system (Applied Biosystems, USA) at the Macrogen, INC, Seoul South Korea.

### Phylogenetic analysis and tree construction

Phylogenetic data were obtained by aligning the nucleotides of different 18S rRNA retrieved from a BLAST algorithm ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)), using the CLUSTAL W program version 1.8 with standard parameters. Phylogenetic and molecular evolutionary analyses were conducted using Mega 6 program (Tamura et al. 2013). All analyses were performed on a bootstrapped data set containing 100 replicates (generated by the program). The GC mol % was generated from NEBcutter, version 1.0, is a program available via a web server (<http://tools.neb.com/NEBcutter>).

### Substrates

Different agricultural wastes (wheat bran, rice straw, soy bean, pomegranate peel, banana peel, orange peel, and potato peel) were used as a carbon source for the production of amylase. The proximate chemical composition for each substrate is presented in Table 1.

All wastes were obtained locally, washed, dried at 70 °C in an oven and milled using blender before use. One substrate further was selected to give the maximum amylase production.

### Fermentation medium and enzyme production

For amylase production, two discs (6 mm in diameter) from culture were inoculated into 250 ml Erlenmeyer conical flasks containing 50 ml of the production medium (g/l): KH<sub>2</sub>PO<sub>4</sub> 1.4; NH<sub>4</sub>NO<sub>3</sub> 10; KCl 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1; FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01; soluble starch 20. The medium was incubated rotary shaker Innova 4080 (New Brunswick, NJ, USA) at 150 rpm and 30 °C for 6 days. The medium was centrifuged at 10,000 rpm for 15 min to obtain a crude enzyme.

### Enzyme assay

Enzyme activity was determined by DNS by a method described by Mandels et al. (1976) using starch as the substrate. The reaction mixture contained the following in a total volume of 2 ml: 1 ml of enzyme extract added 1 ml of 1% soluble starch in citrate phosphate buffer (pH 6.5) and incubated in a water bath at 40 °C for 30 min, and the reaction was stopped by adding 1 ml of DNS reagent (3, 5-dinitro salicylic acid, Sigma-Aldrich Spruce Street, St. Louis, USA) and boiled for 5 min at 80 °C. After stopping the reaction, the tubes were cooled and the absorbance was read at 540 nm. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar.

**Table 1** The proximate chemical composition for different agricultural wastes used as a substrate

Different agricultural wastes	Proximate chemical composition	Reference
Wheat bran	Moisture 8.1–12.7%, protein 9.6–18.6%, Ash 3.9–8.1%, dietary fiber 33.4–63%, total carbohydrate 60–75%, and starch 9.1–38.9%.	(Curti et al. 2013)
Rice straw	Lignin 13.3%, cellulose 31.1%, hemicelluloses 22.3%; Ash and other 33.3%	(Barmina et al. 2013)
Soy bean	Sugar 6%, oil 20%, crude protein 37%, soluble fiber 12%, humidity 9%, ashes 5%, and neutral detergent fiber 11%	(Karr-Lilienthal et al. 2006)
Fresh pomegranate peel powder	Moisture 7.27%, protein 3.76%, ash 4.32%, crude fiber 17.31%, fat 0.85%, total phenol content 18.75 mg/g, antioxidant activity 59.64%, water activity 0.28, and carbohydrate 66.51%.	(Ranjitha et al. 2018)
Fresh banana peel powder	Proteins 0.9%, lipids 1.7%, carbohydrates 59.1% crude fiber 31.7%, water 6 and 8%, minerals: potassium 0.078%, manganese 0.076%, calcium 0.019%, sodium is 0.024%, and iron 0.00061%.	(Anhwange et al. 2009)
Fresh orange peel powder	Ash 4.2%, protein 7.8%, starch 4.5%, fat 2.3%: lignin 1.0%, cellulose 11.1%, and hemicelluloses 12.6%, pectin 51.1%, and moisture 2.9%.	(Hiri et al. 2015)
Potato peel powder	Moisture 11.2%, ash 7.56%, sugar 3.45%, carbohydrate 64.47%, and protein 13.52%.	Arapoglou et al. 2010)

One unit of amylase activity was defined as the amount of enzyme that releases 1  $\mu\text{mol}$  of glucose per minute under the assay conditions.

#### Optimization of culture conditions for amylase production

##### *Effect of agricultural waste on amylase enzyme production*

The enzyme production was studied by using seven different agriculture wastes, e.g., wheat bran, rice straw, soy bean, pomegranate peel, banana peel, orange peel, and potato peel replacing the natural carbon source in the fermentation medium, cheapest and plentifully materials for the amylase production. All agriculture wastes were screened as fermentation substrates for maximum amylase production. The best waste concentration for maximum amylase activity was investigated using different concentrations ranging from 10 to 50 mg/ml.

##### *Effect of incubation time on amylase production*

To measure the optimum incubation time for fungal strain growth and amylase production, the fungal culture was cultivated in production medium and incubated for different incubation time 3, 4, 5, 7, 10, and 12 days at 30 °C on an incubator shaker at 150 rpm.

##### *Effect of temperature on amylase production*

In order to determine the optimum temperature for amylase production by the fungal strain fermentation was carried out at different temperatures by 25, 28, 30, 32, and 35 °C.

##### *Effect of pH on amylase production*

The effect of the pH value was investigated by cultivation the fungal strain in production medium. Media were adjusted to different pH values 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 with NaOH (0.1 N) or HCl (0.1 N).

#### *Partial purification of the enzyme*

Alpha amylase was partially purified by ammonium sulfate fractionation followed by dialysis. The crude enzyme was purified by ammonium sulfate precipitation to 80% saturation in an ice bath. The precipitated protein was collected by centrifugation at 10,000 rpm for 10 min at 4 °C and dissolved in a minimum volume of phosphate buffer (0.1 M; pH 7.0). The enzyme solution was dialyzed at 4 °C against the same buffer for 24 h at 4 °C. The dialysis was concentrated through a freeze dryer and dissolved in a minimum volume of phosphate buffer (0.1 M; pH 7.0).

Amylase activity from partially purified samples was assayed by DNS method. Then, the final concentrated enzyme solution was taken for comparative enzyme entrapment study.

#### *Enzyme immobilization*

##### *Preparation of alginate beads*

Alginate gel beads were prepared according to Awad et al. (2016). Sodium alginate (Alg) was dissolved in distilled water, a volume of enzyme solution and sodium alginate solution was mixed to give a final concentration of 2%, 3%, and 4% (w/v). The calcium alginate gel beads were formed by dropping the mixture alginate solution from a height of approximate 20 cm through a nozzle of 300  $\mu\text{m}$  using syringe into 100 ml solution containing 3%  $\text{CaCl}_2$  (w/v) by a syringe with continuous stirring. The beads were left in the solution for 24 h. The calcium alginate beads containing the enzyme were thoroughly washed with distilled water and used for further studies.

##### *Immobilized enzyme assay*

Enzyme activity was assayed by the DNS method as mention above in the free enzyme method.



**Determination of enzyme immobilization yield**

The immobilization yield was defined here as the yield for an enzyme which was immobilized in the calcium alginate beads and expressed by the following equation:

$$\text{Immobilization yield (\%)} = (\text{activity of immobilized enzyme}/A-B) \times 100$$

Where *A* is the activity of free enzyme added, and *B* is the activity of remaining enzyme in washed water and filtered calcium chloride solution (activity of unbound enzyme). Both *A* and *B* were evaluated for the amount of reducing sugars produced enzymatically in the corresponding solutions.

**Optimization of parameters for enzyme immobilization**

Different sodium alginate concentrations (2–4% w/v) and calcium chloride concentrations (3%) were used during immobilization on  $\alpha$ -amylase to achieve 100% immobilization yield. Also, various factors were studied to evaluate the activity of both the free and immobilized amylase such as incubation time, temperature, pH, and thermal stability of the enzymes.

**Effect of substrate concentration on  $\alpha$ -amylase activity**

The best of substrate concentrate (soluble starch) of immobilized amylase was determined using different starch concentrations (0.25–2.5%).

**Effect of incubation time on  $\alpha$ -amylase activity**

To measure the optimum incubation time for free and immobilized amylase, the reaction mixture at different incubation time (10–60 min) were investigated.

**Effect of temperature and pH on  $\alpha$ -amylase activity**

The optimum temperature of free and immobilized amylase was measured by incubating the reaction mixture of both free and immobilized enzymes at different temperatures ranging from 25 to 70 °C. The effect of pH on enzyme activity was determined by incubation of free and immobilized amylase at optimum temperatures in the pH range of 4–7 using citrate phosphate buffer (pH 4–7).

**Effect of thermal stability of the enzyme**

Enzyme thermal stability of the immobilized enzyme was determined at high temperatures, the enzyme was incubated in the enzyme's buffer solution at different temperatures (25, 30, 40, 50, 60 °C) for different incubation periods (10–60 min), and then they were examined for enzyme activity as mentioned above.

**Results****Molecular identification of the isolate****DNA extraction and PCR amplification**

The DNA content of SS\_RS-NE strain was exposed to PCR via general primers to intensify the ITS1 and ITS4 regions among the minor and great nuclear rDNA, counting the 18S rDNA. The DNA fragment of 490 bp was improved by these primers.

The nucleotide sequence (490 bp) of strain *Aspergillus terreus* SS\_RS-NE was blasting the sequence with the obtainable GenBank sources by NCBI-BLAST ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) to associate the SS\_RS-NE isolate through those of adherent *Aspergillus* sp. microorganisms. The outcomes exhibited that the highest sequence correspondence types (99%) with *Aspergillus terreus* strains.

**Phylogenetic analysis and GC%**

The phylogenetic tree (Fig. 1) showed that strain SS\_RS-NE is most nearly correlated to *Aspergillus terreus*. Consequently, it was suggested a name *Aspergillus terreus* SS\_RS-NE. The GC% is one of numerous universal structures used to describe fungal genomes. The GC content of the genomic DNA was 59 mol% for SS\_RS-NE isolate was achieved from the phylogenetic examination.

**GenBank accession number**

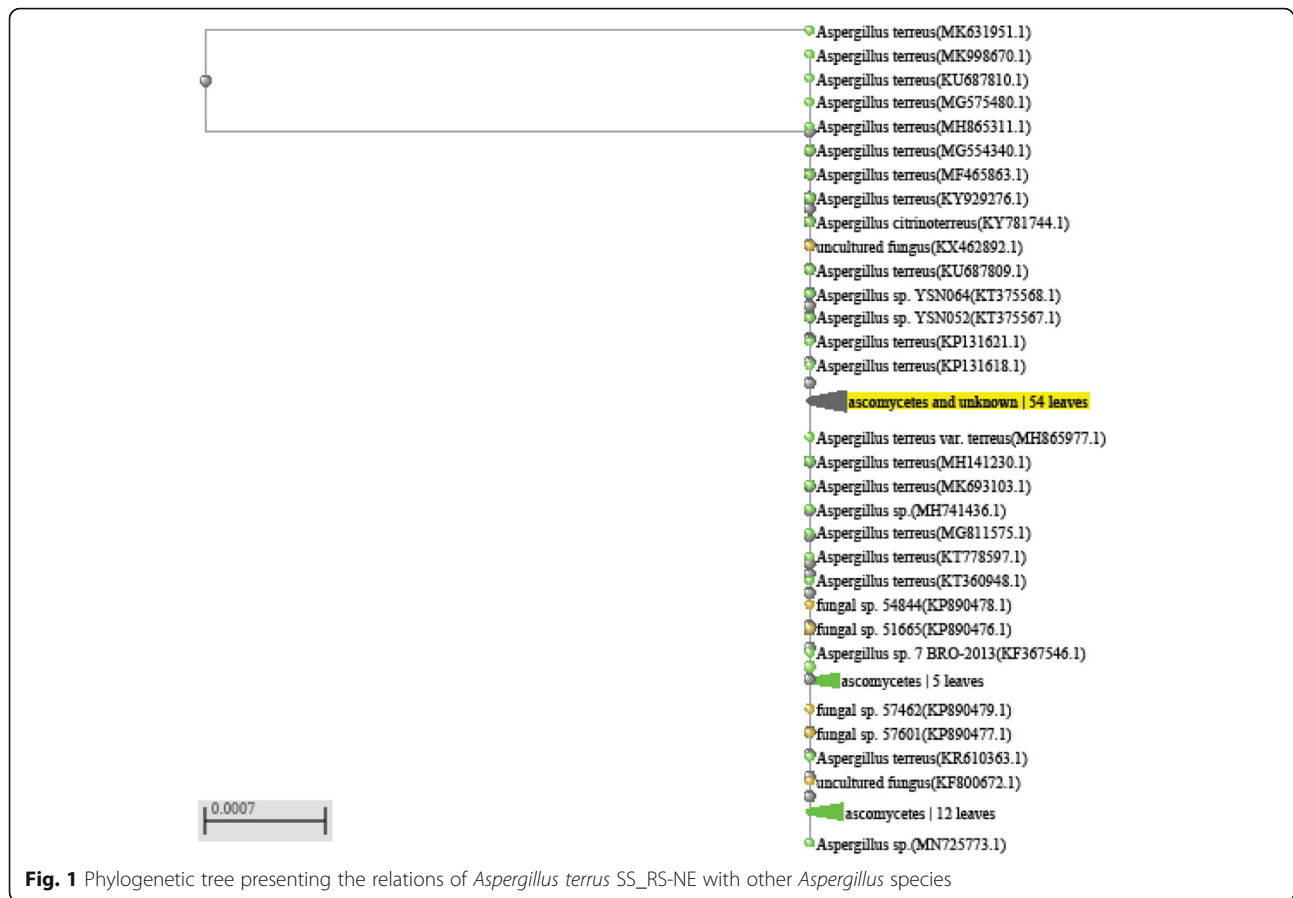
The nucleotide sequences of 18S rRNA gene of *Aspergillus terreus* SS\_RS-NE have been placed in GenBank under accession number MN901491.

**Effect of different agricultural wastes on amylase production by *Aspergillus terreus***

Various agricultural wastes such as wheat bran, rice straw, soy bean, pomegranate peel, banana peel, orange peel, and potato peel were used as cheap and most economical carbon source for amylase production by *Aspergillus terreus*. The results in Fig. 2 showed that the maximal amylase activity was noticed using pomegranate peel (339 U/ml) as the substrate, soybean (273.59 U/ml), followed by banana peel (232.35 U/ml). While other wastes, e.g., wheat bran, orange peel, potato peel has moderate to low activity. On the other hand, the minimum amylase activity was recorded in rice straw (77.53 U/ml). Thus, pomegranate peel waste was carefully chosen for additional experiment on amylase production.

**Effect of different pomegranate peel waste concentrations on amylase production**

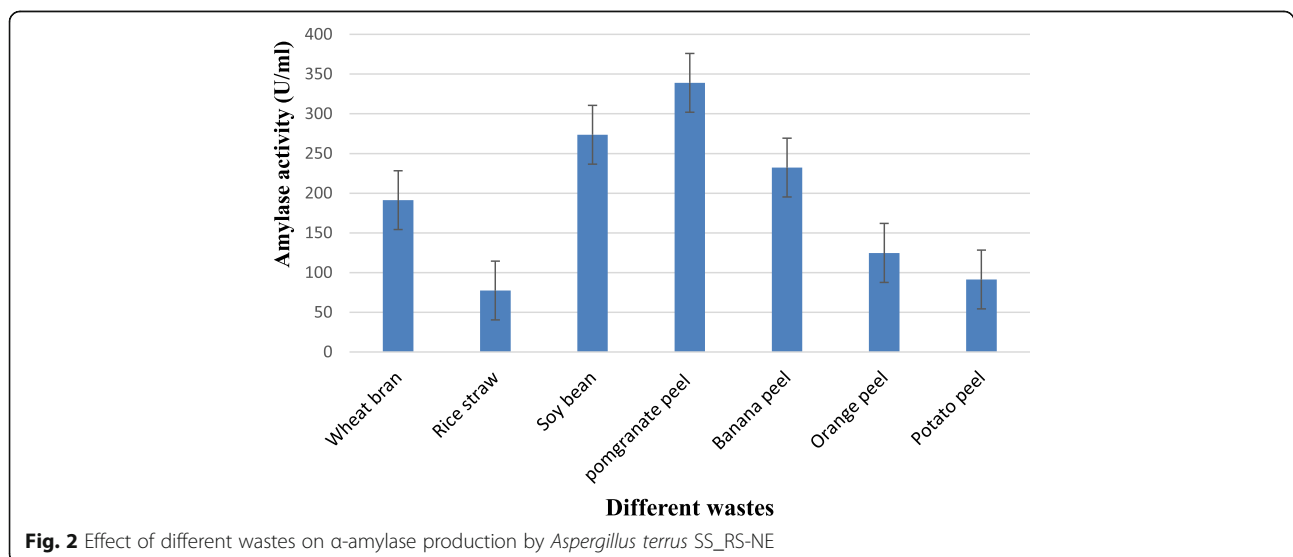
The effect of different concentrations of pomegranate peels on the  $\alpha$ -amylase activity by *A. terreus* was studied. The results illustrated in Fig. 3 showed that



*A. terreus* produced the highest  $\alpha$ -amylase activity (340.69 U/ml) at 1.5% pomegranate peel. Further decrease or increase in the pomegranate peels concentration decreased  $\alpha$ -amylase production. Most likely, that the substrate concentration influences  $\alpha$ -amylase creation.

**Effect of different incubation time on  $\alpha$ -amylase production**

The effect of different incubation time (3–12 days) on amylase production by *A. terreus* using pomegranate peel 1.5% as carbon source at optimum temperature and pH were investigated. As shown in Fig. 4a, amylase



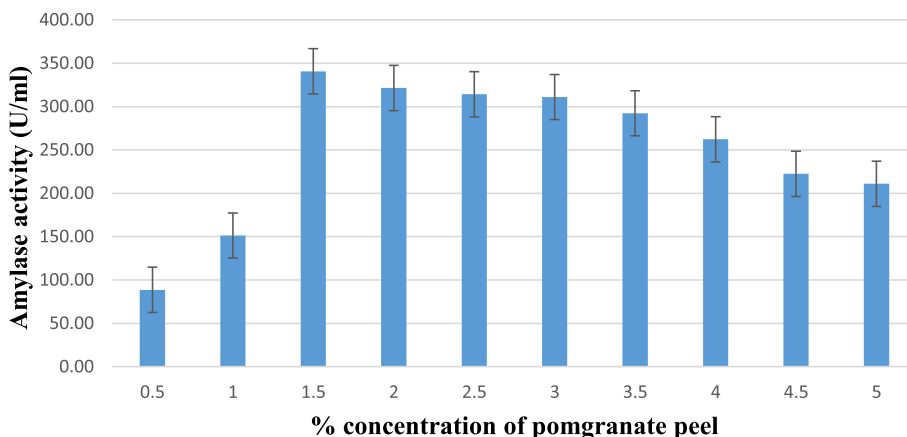


Fig. 3 Effect of different concentration of substrate on  $\alpha$ -amylase produced by *Aspergillus terreus* SS\_RS-NE

activity increased gradually with increasing of incubation time till reaching the maximum activity of 343.8 U/ml on 5 days incubation time, then amylase activity gradually decreased by increasing the incubation time. This may be due to the depletion of essential nutrients required for the growth and enzyme production.

**Effect of different pH values on  $\alpha$ -amylase production**

The effect of different pH of the medium (3.5–7) on the amylases production by *A. terreus* using pomgranate peel 1.5% was studied. The results in Fig. 4b indicated that the maximal enzyme activity of 345 U/ml was obtained at pH 6.0, over this pH value, the amylase activity gradually decreased.

**Effect of different temperature on amylase activity**

The influence of diverse incubation temperature on the amylase production from *A. terreus* using pomgranate peel 1.5%, for selecting the optimum incubation time and pH value was studied. The results in Fig. 4c showed that

the maximal amylase yield of 348.5 U/ml was observed at 30 °C, below 30 °C, amylase activity was decreased. Vidyalakshmi et al. (2009) denoted that a temperature is one of the significant elements, which powerfully affect  $\alpha$ -amylase activity by fermentation procedure.

**Partial purification of crude amylase**

The crude enzyme that produced from *A. terreus* using pomgranate peel waste 1.5% was partly refined by 80% ammonium sulfate and dialysis. The outcomes in Table 2 indicated that the amylase yield of 1246 and 2411 U/ml was obtained from 80% ammonium sulfate and dialysis, respectively, in comparison to crude amylase of 349 U/ml.

**Amylase immobilization**

**Effect of different concentration of sodium alginate on immobilized enzyme yield**

The immobilization yield of enzyme is influenced by on Na alg. concentration. So, 2–4% Na alg. was used for

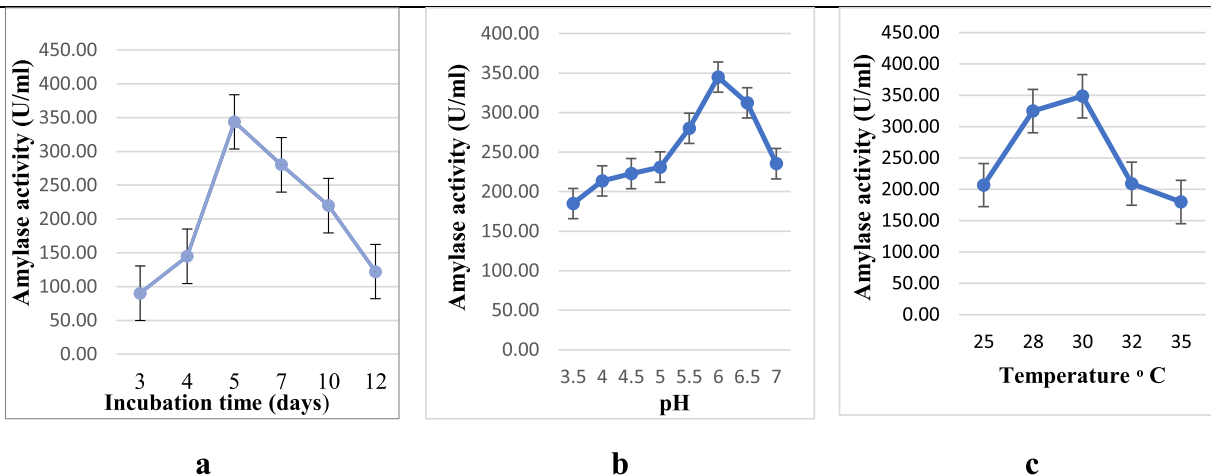


Fig. 4 Effect of different incubation time (a), different pH (b), and different temperature (c) on  $\alpha$ -amylase produced by *Aspergillus terreus* SS\_RS-NE

**Table 2** Partial Purification of amylase enzyme produced by *Aspergillus terreus*

Steps of partial purification	Amylase activity (U/ml)
Crude enzyme	349
Precipitate by ammonium sulfate (80%)	1246
After dialysis	2411

immobilized amylase preparation. The results illustrated in Fig. 5 indicated that immobilized amylase with 2% sodium alginate showed the highest immobilization yield (92.8%) while 3% sodium alginate gave the yield of 86.6%. A lower immobilization yield was obtained by 4% sodium alginate (22%). Immobilization yield decreases with the increasing in the concentration of Na alg. The pore size of the beads would be such that substrate and product can simply diffuse in and out of the alginate gel matrix, but the amylase would retain in the micro environment of beads. Lower concentration of Na alg solution, the pore size of the beads will be increased and consequently escape of the enzyme from the beads will rise and vice versa (Riaz et al. 2009).

#### Characterization of amylase immobilized

##### *Influence of substrate concentration on immobilized amylase*

The influence of substrate concentration on immobilized amylase activity using different concentrations of starch (0.25–2.5%) at 40 °C for 30 min, pH 6.5 was studied. The results illustrated in Fig. 6 showed that the activity of immobilized amylase yield gradually increase till reached its maximum activity of 2522.5 U/ml at 2% starch, and then the activity was declined.

##### *Effect of different reaction time on the activity of free and immobilized amylase*

The effect of different reaction time (10–60 min) for both free and immobilized amylase was investigated with

2% starch. The results in Fig. 7a indicated that the enzyme activity of both free and immobilized amylase was increased with the increasing of enzyme reaction time and substrate. Consequently, the optimum reaction time for free and immobilized amylase was found to be 40 min and 10 min of the reaction, over this reaction time, both free and immobilized amylase activity was decreased.

##### *Effect of different pH values on the activity of free and immobilized amylase*

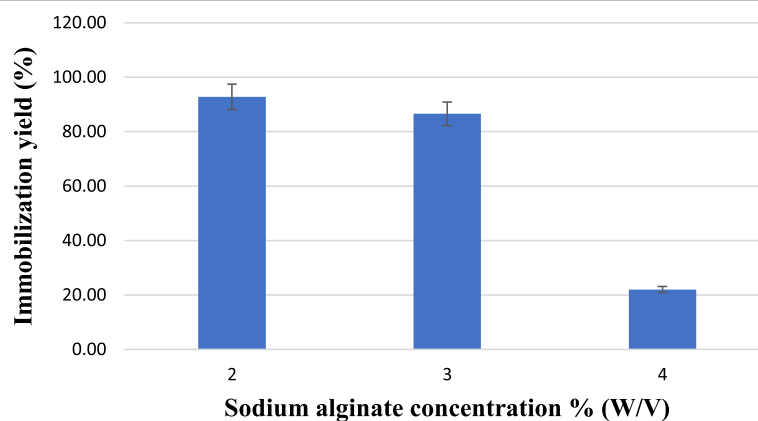
The pH is one of the major parameters capable of shifting enzyme activities in the reaction mixture. Immobilization usually results in a shift of optimum pH due to conformational changes in enzymes. The activity of both free and immobilized amylase was investigated at different pH (4–7) with 2% starch. Increasing in pH has marked effect on free and immobilized amylase enzyme as shown in Fig. 7b. The results indicated that the optimum pH for free and immobilized amylase enzyme was 5 and 6.5, respectively.

##### *Effect of different temperature on free and immobilized amylase*

The activity of free and immobilized amylase was investigated at different temperatures (25–70 °C). The results presented in Fig. 7c showed that, the activity of free and immobilized amylase increase with the increasing the temperature till reached its maximal yield at the optimum temperature of 50 °C and 60 °C for free and immobilized enzyme, respectively.

##### *Thermal stability of immobilized amylase*

The thermal stability of the immobilized amylase was studied by incubating the enzyme at different temperature ranging from 25 to 60 °C and the residual activity was measured according to the standard assay procedure at different incubation time of 10–60 min. As

**Fig. 5** Effect of concentration of sodium alginate on amylase immobilization yield



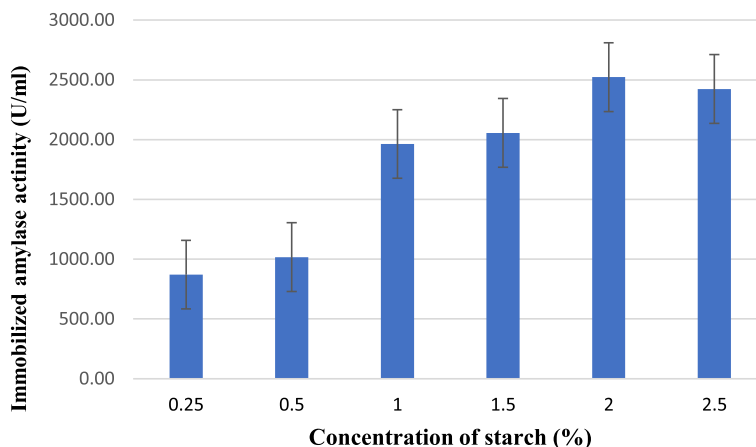


Fig. 6 Effect of concentration of substrate on the activity of immobilized amylase

shown in Fig. 8 the immobilized amylase was stable at 60° C for 20 min.

**Discussion**

Molecular identification of the isolate was achieved by specific primers that improved a DNA piece of about 490 bp. These outcomes were in covenant with Rasul et al. (2007) who established that these primers are precise for molds and improved a DNA fragment of about 579 bp with some fungi. The GC contented of the genomic DNA was 59mol% for SS\_RS-NE isolate. These outcomes were in agreement with Nakase and Komagata (1971) who stated the GC contented of mold varieties from 31.5 to 63%. This variety differed for each class. The compositional variety likewise decays from classes and subclasses to types and sort.

The production of amylase enzyme from fungi using agricultural waste has been investigated by

several authors. The maximal amylase activity of 339 U/ml was obtained using pomegranate peel as shown in Fig. 2. These results were in covenant with Singh et al. (2014) who found that the maximal amylase of 335.4 U/ml from *Aspergillus fumigatus* NTCC1222 using pomegranate peel. On the other hand, these results were greater than the amylase yield of 259 U/ml and 112 U/ml that obtained by *A. niger* and *Bacillus amyloliquefaciens* using banana waste wheat bran, respectively (Cyprian et al. 2017; Mojumdar and Deka 2019).

The highest α-amylase yield of 340.69 U/ml at 1.5% pomegranate peel as noticed in Fig. 3. Similar findings were recorded by Saleh et al. (2011) and Ali et al. (2017) who indicated that the uppermost amylase yield was acquired at 5% and 3% of mandarin peel by *Trichoderma harzianum* and *Aspergillus flavus* AUMC 11685, respectively.

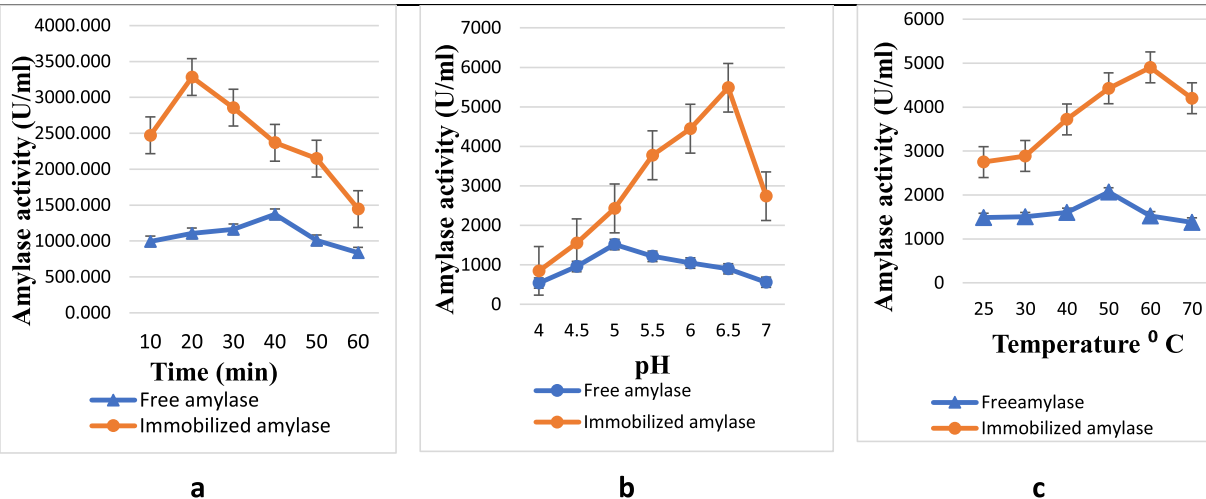
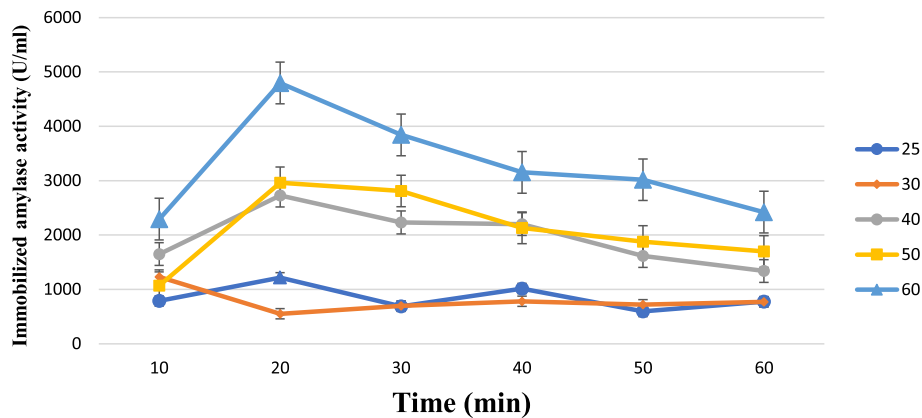


Fig. 7 Effect of different reaction time (a) different pH (b), and different temperature (c) on the activity of free and immobilized amylase



**Fig. 8** Thermal stability of immobilized amylase by *Aspergillus terreus* SS\_RS-NE

The effect of different incubation time on the enzyme activity was investigated. The fungal strain produced the maximum amylase activity of 343.8 U/ml on 5 days incubation time (Fig. 4a). Comparable consequences were informed by Singh et al. (2014) who denoted that the enzyme activity of 262.3 U/ml by *A. fumigatus* NTCC1222 increase with the increasing in the cultivation period, accomplishment the maximal on the 6th day, the enzyme activity was declined on the 7th and 8th day of incubation period. Also, Ali et al. (2017) found that the highest  $\alpha$ -amylase yield of 33.52 U/ml by *A. flavus* AUMC 11685 using mandarin peel was obtained on the 4th day.

Among, the pH of medium assumes a significant role in inciting morphological variations in molds and in the enzyme excretion (Acourene et al. 2013). The maximal enzyme activity of 345 U/ml was obtained at pH 6.0 (Fig. 4b). In contrast to our outcomes, Singh et al. (2014) who stated the maximum amylase production by *A. fumigatus* NTCC1222 was 339.1 U/ml at an initial pH 6.0. These results can be compared with those informed by Oyeleke et al. (2010); Pasin et al. (2014) and Ali et al. (2017) they observed that the best initial pH for amylase production ranged from 5 to 5.5 by *A. niger*, *A. japonicus* and *A. flavus* AUMC 11685, respectively.

Temperature is one of the significant elements, which emphatically influence  $\alpha$ -amylase formation by maturation method (Vidyalakshmi et al. 2009). The optimum temperature that gave the maximal amylase yield of 348.5 U/ml was observed at 30 °C (Fig. 4c). These results were in accordance with several authors who observed that amylase activity was optimal at 30 °C by *A. oryzae*, *Penicillium fellutanum*, *Aspergillus* sp. JGI 12 (Ramachandran et al. 2004; Kathiresan and Manivannan 2006; Alva et al. 2007).

The amylase activity was 3.5- and 7-folds were obtained from 80% ammonium sulfate and dialysis, respectively (Table 1). The same consequences were stated by Singh et al. (2014) who found that the specific activity

of the partially purified enzyme by ammonium sulfate precipitation and dialysis was 7- and 20-fold purification, individually. As well as, Devi et al. (2012) and Sidkey et al. (2011) reported that 50% and 60% ammonium sulfate gave the highest enzyme activity.

The highest immobilized enzyme yield of 92.8% achieved with 2% sodium alginate (Fig. 5). The consequences of this work were in concurrence with Mahajan et al. (2010) who indicated that immobilization proficiency and dispersal of substrate into the beads were approved by 1% sodium alginate. Different concentrations (1.5–5.0%) of sodium alginate were utilized for the preparation of immobilized enzyme. The amylase was successfully immobilized by entrapping in alginate. These results improve the finding that told the increasing of the concentration of sodium alginate prevents the entrance of substrate into the beads that directed to the lower immobilization efficacy (Dey et al. 2003; Riaz et al. 2009).

The maximum immobilized amylase yield of 2522.5 U/ml was enhanced at 2% starch (Fig. 6). Our results were agreed with Hemanchi and Sanjay. (2019) who found that 1% starch gave the highest immobilized enzyme activity of 2736 U/ml. Also, Zufahair et al. (2017) indicated that the optimum substrate concentration of immobilized amylase was at concentrations of 1.5–2.5%. This improved necessity of the substrate upon immobilization has been described by Riaz et al. (2007) who found that dissemination of high molecular weight polysaccharide as starch huge will be constrained with the matrix; its diffusional opposition from the bulk solution to the micro environs of an immobilized enzyme can confine the reaction rate.

The enzyme activity of both free and immobilized amylase was increased with the increasing of enzyme reaction time and substrate (Fig. 7a). The obtained results were in accordance with Devi et al. (2012) and Riaz et al. (2015) they mentioned that the maximal immobilized  $\alpha$ -

amylase activity was recorded at 10 min. Beyond 10 min, immobilized  $\alpha$ -amylase activity was decreased.

Increasing in pH has marked effect on free and immobilized amylase enzyme. The optimal pH of 5 and 6.5 was noticed for free and immobilized amylase enzyme (Fig. 7b). These results were most close to the data obtained by Hemanchi and Sanjay. (2019) who mentioned that pH values of 5.8 and 6.8 were optima for free and immobilized  $\alpha$ -amylase. On the other hand, our results were in disagreement with Talekar and Chavare (2012) who indicated that the optimal pH values for free and immobilized amylase was 7 and 5.5, respectively. The change in the optimum pH of immobilized  $\alpha$ -amylase due to conformational changes in enzymes; variation in acid and base amino acid side chain ionization in the microenvironment round the active site (Talekar et al. 2010; Prakash and Jaiswal 2011).

The maximal yield for free and immobilized amylase was obtained at 50 °C and 60 °C (Fig. 7c). These results were in agreement with Talekar and Chavare (2012); Devi et al. (2012), and Hemanchi and Sanjay (2019) who found that the maximum activity of free and immobilized  $\alpha$ -amylase was ranged from 50 °C to 70 °C using calcium alginate beads.

The immobilized amylase was stable at 60 °C for 20 min (Fig. 8). Similar results were recorded by El-Banna et al. (2007) who found that free and immobilized  $\alpha$ -amylase has thermal stability at 60 °C for 10 min and were in coincidence with Hemanchi and Sanjay (2019) who mentioned that 40% relative immobilized  $\alpha$ -amylase activity was obtained by exposing the beads to 60 °C for 60 min. The concentration of 50% of the free enzyme reserved its thermal stability up to 50 °C compared to 60 °C to the immobilized enzyme preparation (Foukia et al. 2016).

## Conclusions

Amylases are amongst the most important hydrolytic enzymes that are used in numerous industrial uses reaching from food to pharmaceuticals. Marine fungal was isolated from red sea water at Sharm El-Sheikh region and genotypic identified as *Aspergillus terrus* SS\_RS-NE (MN901491) and it has the ability to produce amylase using pomegranate peel waste with a yield of 339 U/ml. The crude enzyme was partially purified by ammonium sulfate followed by dialysis. The maximal immobilized amylase activity of 2522.5 U/ml was obtained under optimized some of culture conditions and medium nutrient parameters.

## Abbreviations

PDA: Potato dextrose agar; PCR: Polymerase chain reaction; DNS: 3, 5-dinitro salicylic acid; Rpm: Round per minute; Sod Alg: Sodium alginate; bp: Base pair; GC: Guanine and cytosine; Pp: Pomegranate peel

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## Ethical approval and consent to participate

Not applicable

## Authors' contributions

NA: selection of microorganism, enzymes assay, optimization conditions, writing these parts. Fabricated the XYZ of graphs for the experiments. AE: enzyme immobilization, enzyme assay, writing immobilization part. HM: strain identification, writing identification part, formatting, and revising the manuscript. All authors have read and approved the final article.

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All information generated or analyzed during this work are incorporated in this manuscript.

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## Competing interests

The authors declare that they have no competing interests.

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