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Isolation, Molecular Identification, Mutation Induction of Egyptian Fungus *Aspergillus niger NM-NRC* and Medium Optimization Using Response Surface Methodology for Pectinase Hyper Production

Nagwa M. Abd El-Aziz[1] and Nora N. El-Gamal[2]

[1]Microbial Genetics Department, Biotechnology Research Institute, National Research Centre, 33 El Buhouth ST, Postal code 12622, Dokki, Cairo, Egypt.
[2]Microbial Chemistry Department, Biotechnology Research Institute, National Research Centre, 33 El Buhouth ST, Postal code 12622, Dokki, Cairo, Egypt.

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ABSTRACT

A class of enzymes called pectinases aids in the digestion of pectic substances. In the food business, it's commonly used to produce and clarify juices and wines. The aim of this study was to isolate, assess, and examine pectinase enzymes from fungi derived from various soil specimens. A total of 15 fungal isolates were first screened from 10 distinct soil samples. Out of them, only one exhibited the most pronounced pectinolytic activity was isolate No. 1 exhibited the greatest enzyme output after a 3 days incubation period, with a substrate citrus pectin concentration of 1% and a temperature of 30°C. The enzyme production was measured at 51.47 U/mg. Internal transcribed spacer (ITS) sequencing and PCR were used to confirm the molecular identity of isolate No. 1, which was determined to be *Aspergillus niger* NM-NRC. The results were deposited in the NCBI database under the accession numbers OQ600201. In order to increase the efficiency of pectinase production in this strain, a series of mutagenesis techniques were employed, including ultraviolet (UV) radiation, ethidium bromide (EtBr), and hydrogen peroxide (H_2O_2). A number of mutants were produced, and the mutant H-34 from *Aspergillus niger* NM-NRC was shown to be the most effective in breaking down pectin, with a pectinase activity of 113.43 U/mg. Response Surface Methodology (RSM) was used to establish the ideal conditions for pectinase expression in the *Aspergillus niger* NM-NRC mutant H-34. The optimal culture conditions for achieving the greatest pectinase-specific activity of 164.45 U/mg were a temperature of 30°C, pH of 9, 48 hours of incubation, and a pectin concentration of 2%. Additionally, 1.5% glucose and 1.5% beef extract were used as carbon and nitrogen sources, respectively. In conclusion, the strain *Aspergillus niger* NM-NRC mutant H-34 has been determined to be highly effective in producing pectinase on a large scale for commercial use.

Keywords: pectinase, internal transcribed spacer regions, ultraviolet, Hydrogen peroxide, ethidium bromide, response surface methodology.

1. Introduction

Higher plants' primary cell wall and middle lamella both contain significant amounts of pectin. Pectins are acidic heteropolysaccharides with a high molecular weight, mainly composed of α (1−4) linked d-galacturonic acid residues (Kavuthodi and Sebastian, 2018). There are three main kinds of pectic polysaccharides that have been identified, and they are all partially or mostly composed of d-galacturonic acid. Rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), and homogalacturonan (HG) are their names (Alkorta *et al.,* 1998; Carrasco *et al.,* 2019). A class of enzymes known as pectinases is categorised based on how they break down pectic substances. Methoxy groups, for instance, are eliminated from strongly or partially esterified galacturonan by methylesterases. Exopoly-glacturonases and endopolygalacturonases, which release galacturonic or digalacturunic acid residues

Corresponding Author: Nagwa M. Abd El-Aziz, Department of Microbial Genetics, National Research Centre, Dokki, Giza, 12622, Egypt. E-mail: nagwamkh@gmail.com

from the nonreducing end of homogalacturonan, are two types of polygalacturonases that catalyse the hydrolysis of glycosidic linkages in an erratic manner (Cornuault *et al.,* 2018; Pedrolli *et al.,* 2009). Pectinases, also known as pectinolytic enzymes, are further categorised based on their substrate and mode of action. These include polygalacturonases, which are further divided into endo-polygalacturonases (EC. 3.2.1.15) and exo-polygalacturonases (EC. 3.2.1.67); lyases, which are further divided into pectatelyases (EC. 4.2.2.9 and 4.2.2.2) or pectin lyases (E.C. 4.2.2.10) and pectin methylesterases (E.C. 3.1.1.11). Combining different types of pectinases with other enzymes like cellulases and hemicelullases is advised because these enzymes can break down different regions of the polymer, maximising the amount of pectin that is broken down in a variety of raw materials, like citrus juice processing (Oumer and Abate, 2018; Garg *et al.,* 2016). Studies have shown that the market for microbial pectinase, which makes up 25% of the world's sales of industrial and culinary enzymes, is constantly growing (Oumer, 2017). Enzymes are also part of a well-established global market that is expected to grow to USD 6.3 billion by 2021 (Oumer and Abate, 2018). Fungal microorganisms are a possible source of enzymes. Several extracellular enzymes produced by fungi have a unique role in the breakdown of organic materials. Among them are the pectinolytic enzymes that plants release to disintegrate the central lamella, allowing fungi to insert their hyphae and draw nutrients from the plant (Gummadi and Panda., 2003; Hoondal *et al.,* 2002). Apart from fungi, numerous other creatures such as bacteria, insects, worms, and protozoans naturally produce pectinolytic enzymes (Khairnar *et al.,* 2009). *Erwinia spp, Aspergillus spp.. Penicillium spp*. and *Bacillus spp*. have all been widely used in the commercial manufacture of pectinases (Oumer, 2017; Dhital *et al.,* 2014). Pectinases are essential to the food industry. These enzymes are used for extracting fruit juice, clarifying wine, concentrating and fermenting tea, cocoa, and coffee, extracting vegetable oil, making jam and jellies, and pickling (Barman *et al.,* 2015; Kubra *et al.,* 2018). Furthermore, these enzymes are used in the pulp and paper sectors, as well as in the extraction of oil, the treatment of wastewater, the bleaching, bio-scouring, retting, and degumming of plant fibres, additives for poultry feed, protoplast fusion technology, and the production of bioenergy.(Gummadi and Panda., 2003; Kubra *et al.,* 2018). The way that enzymes break down biomolecules is dependent on a number of factors, including the type of microorganisms present, the fermentation environment (pH, length of incubation or cultivation, carbon and nitrogen source, substrate types and concentrations, temperature, agitation, and use of different enzyme preparations). (Koirala *et al.,* 2014; Patidar *et al.,* 2018). It has always been believed that research is crucial when it comes to the deployment of novel enzymes with desired biochemical and physicochemical properties and cheap production costs in commercial operations. The goals of this work were to: (1) separate and screen pectinase-producing fungi from soil samples; and (2) enhance the chosen strain of pectinase-producing fungus by appropriate physical and chemical mutagenesis, taking into account all the benefits. (3) The optimization of diverse factors to achieve optimal enzyme synthesis and the assessment of enzyme activity using several parameters.

2. Material and Methods

2.1. Collecting soil samples

Soil samples were gathered from several areas throughout Cairo City, Egypt. These sites were abundant in rotten fruits, agro-industrial wastes, fruit pulp, composts, decaying leaves, and organic fertilizers. A total of 10 soil samples were collected from a depth of 20 cm and placed in sterile zip-lock bags. These samples were then brought to the laboratory for analysis (KC *et al.,* 2020).

2.2. Isolation and primary screening of pectinolytic fungi

The soil samples were analyzed using Potato Dextrose Agar (PDA, HiMedia) to isolate pectinolytic fungi. To isolate the microorganisms, a five gram soil sample was put into 45 milliliters of distilled water. The sample was then serially diluted up to a dilution factor of 10^{-6}. For the purpose of dilution, a volume of 0.1 mL was introduced onto PDA agar plates using the spread plate method. After that, the plates were incubated for three to five days at 30°C. For replication, a variety of colonies were selected and moved on two different PDA plates. The plates were then placed in an incubator at a temperature of 30°C for a period of 3–5 days. Fungal colonies displaying different morphology were carefully chosen and subjected to repeated subculturing in order to obtain a pure culture through point inoculation. The initial fungal isolates were placed on a specialized agar medium called pectinase screening agar medium (PSAM) and kept in an incubator at a temperature of 30°C for a period of 3-5 days incubation. The

PSAM solution consists of the following components, measured in grams per liter (g L^{-1}): $(NH_4)_2H_2PO_4$, 3.0; KH_2PO_4, 2.0; K_2HPO_4, 3.0; $MgSO_4$, 0.1; citrus pectin, 10.0; and agar, 25.0. The acidity level of the media was modified to a pH of 5. Following incubation, the plates were immersed in a solution of 1% (w/v) cetyltrimethylammonium bromide (CTAB) and kept at a temperature of 37 ◦C for a duration of 15 min. The colonies were preserved for further examination since the existence of a hydrolysis zone around them indicated the fungus's pectinolytic activity (Carrasco *et al*., 2019; Khairnar *et al*., 2009).

2.3. Submerged fermentation for enzyme production and quantitative assay of pectinase producing fungal isolates

Submerged fermentation (SmF) was used in the enzyme production process. 50 mL of PSAM broth were made in a conical flask, and after that, it was chilled and sterilized using the previously mentioned procedure (Hankin and Anagnostakis, 1975). One millilitre of the pre-fermenter's inoculum was then added. For a period of three to five days, the flasks were kept in a shaking incubator and agitated at 150 revolutions per minute (rpm) at 30 degrees Celsius (Kashyap *et al.,* 2000). Following previously reported methods, the dinitro salicylic acid (DNS) test was used to measure the pectinase activity in the fermented broth (Miller, 1959). Using a pipette, two millilitres of the fermented broth were placed into a sterile tube, and centrifugation was applied for twenty minutes at 8000 revolutions per minute. The liquid fraction obtained from centrifugation served as the raw enzyme and was used for analysis. One millilitre of the raw enzyme and one millilitre of 3% pectin were combined in a sterile tube. After that, the mixture was incubated for fifteen minutes at fifty◦C. To stop the hydrolysis reaction, 1 mL of the DNS (Dinitro salicylic acid) reagent was added after the incubation period. After that, the mixture was thoroughly mixed and let to sit in a hot water bath for half an hour in order to speed up the colour development. Accompanying the enzyme and substrate blank runs, a spectrophotometer was used to detect the absorbance at 540 nm. One millilitre of 0.5% pectin, one millilitre of sodium acetate buffer (0.1 M, pH 4.2), and two millilitres of DNS reagent were combined to provide a control (KC *et al.,* 2020). The amount of enzyme that releases one µmol of galacturonic acid per hour under standard test circumstances is defined as one unit of enzyme activity.

2.4. Molecular identification of internal transcribed spacers (ITS) gene and PCR amplification of fungal isolate

According to group directions given by QIAGEN's QIAamp DNA Mini Kit and the GeneJET Genomic DNA Purification Kit in order to isolate the genomic DNA. The polymerase chain reaction (PCR) was conducted utilizing the forward primer. ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) by utilizing the forward and reverse primers, we can amplify a specific region of the nuclear ribosomal gene cluster that includes the internal transcribed spacers (ITS) gene (Singh and Saxena, 2010). The PCR protocol consisted of an initial step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds. A final elongation step was conducted at a temperature of 72°C for duration of 5 minutes. The PCR result was observed on a 1% agarose gel using a 100-bp ladder DNA marker from Invitrogen, located in California, USA (Altschul *et al.,* 1990). Afterwards, the purified PCR products underwent sequencing, and the resulting sequence data were analyzed using the basic local alignment search tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast).The alignment was performed using the MAFFT alignment server for phylogeny (https://mafft.cbrc.jp/alignment/ server/phylogeny.html) (Rozewicki *et al.* 2019).

2.5. Hydrogen peroxide, ultraviolet, and ethidium bromide Mutagenesis

The strain *Aspergillus niger* NM-NRC, which has the normal genetic makeup, was grown in a liquid PDA medium at a temperature of 30°C for duration of 3-5 days. Afterwards, a 10 ml sample of the culture was centrifuged at a force of 9000g and a temperature of 4°C for 10 minutes to separate the cell biomass. The cellular biomass pellet was subsequently reconstituted in a 10 ml solution of sterile saline (0.9%). In order to initiate mutagenesis through UV radiation, a sterile culture of 4 ml was exposed to UV light for duration of 60 minutes. This was done using a 15 W lamps UV-dispensing cabinet, which emitted approximately 90% of their energy at a wavelength of 265 nm. Afterwards, the culture was placed in darkness overnight to avoid photo reactivation (Sanaa, 2018). To induce mutagenesis, the plates were treated with 10 mg/ml of ethidium bromide and hydrogen peroxide was supplemented with 5

µl of 30% (v/v) H_2O_2. (Akbar *et al.,* 2015; Akbar *et al.,* 2013; Kamalambigeswari *et al.,* 2018) and kept in a controlled environment at a temperature of 30°C for a duration of 60 minutes. After subjecting the cells to all mutagenesis treatments, they were gathered by centrifugation at 2800*xg* for 15 minutes. The material was then purified using sterile saline solution and thereafter transferred onto agar plates containing PDA media. Subsequently, the plates were placed in an incubator set at a temperature of 30ºC for duration of 3-5 days. The surviving colonies were further examined for pectinase-specific activity to discover mutants that exhibit great efficiency in producing pectinase (Duarte *et al.*, 2011).

2.6. Optimization of the fermentation conditions for maximum enzyme production

The optimization of growing conditions for pectinase synthesis in mutant *Aspergillus niger* NM-NRC was carried out through two phases of statistical design research. The statistics software Design-Expert® 6.0.8. The experimental design and analysis software was developed by Stat-Ease, a company based in Minneapolis, MN, USA. Initially, the most suitable nitrogen sources (tryptone, peptone, beef extract, malt extract and yeast extract) was determined and carbon sources (sucrose, glucose, lactose, fructose and xylose) for the production of pectinase, and concentration of these sources was 0.5%. The second step involved evaluating each independent variable using response surface methodology. The factors included in the study were temperature (25°C, 30°C, and 35°C), pectin content (1%, 2%, and 3%), pH levels (5, 7, and 9), incubation length (24, 48, and 72 hours), carbon source (glucose), and nitrogen source (beef extract). A Box-Behnken design was used to evaluate these aspects at three levels, **presented in Table 1**. The response of pectinase activity was evaluated using 86 experimental designs (Gupta and Singh, 2013; Singh and Saxena, 2010). The general model equation $Y = \beta_o+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_{12} + \beta_{22}X_{22} + \beta_{33}X_{32} + \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{23}X_2X_3$ The assessment of pectinase activity was conducted using variance analysis, and Fisher's F test ($p < 0.05$) validated the significance of each coefficient. Three-dimensional (3D) contour plots to visually represent the quadratic models, and conducted an analysis of variance (ANOVA) on the response surface methodology (RSM) data collected for pectinase production. The experiments were conducted thrice for each trial (Tiwary and Gupta, 2010).

Table 1: Experimental factors and level of minimum and maximum range for statistical screening using Box-Behnken design

Factors	Independent Factor	Unit	Type	Range level		
				Minimum (−1)	Medium (0)	Maximum (+1)
X1	**Incubation time**	Hours	Factor	24	48	72
X2	**pH**	+H	Factor	5	7	9
X3	**Glucose (carbon source)**	% (w/v)	Factor	0.5	1.5	2.5
X4	**Beef extract (nitrogen source)**	% (w/v)	Factor	0.5	1.5	2.5
X5	**Pectin concentration**	% (w/v)	Factor	1	2	3
X6	**Temperature**	° C	Factor	25	30	35
X7	**pectinase activity**	U/mg	Response			

3. Results

3.1. Isolation and primary screening of pectinase-producing fungal isolates

A total of 15 fungal isolates were obtained and extracted from soil samples in Cairo, Egypt, and subsequently purified. The isolates were screened for their pectin degradation capability using the plate assay method in the pectinase screening agar medium (PSAM) with 1% pectin as the only carbon source. The diameter of each hydrolysis zone in the plate was measured and recorded after the plates were exposed to a 1% CTAB solution for 15 minutes at 37ºC. This was done to determine the probable fungal isolate. Out of the 15 fungal isolates, 13 isolates exhibited distinct areas, indicating the presence of pectinase activity and classifying them as producers of the pectinase enzyme. The fungus capable of hydrolyzing pectin in the clear zone was tested for its pectinase-specific activity.

3.2. Quantitative pectinase assay of fungal isolates

The fungal isolates were assessed for their ability to produce pectinase and their specific enzymatic activity. The results indicated a broad spectrum of pectinase-specific action. Among all the isolates, only one No. 1 shown the highest degree of pectinase-specific activity, with recorded value of 51.47 U/mg. The protein levels of all isolates were also evaluated at A_{280} to conduct an additional test for pectinase activity. The isolate that had the highest protein content in this study was identified as isolate No. 1. That had protein contents of, 1.17±0.02 mg/ml, in a basic medium. The NM-NRC isolate No. 1, **as depicted in figure 1**, possesses the capacity to completely degrade 1% pectin within a time frame of 3 days incubation.

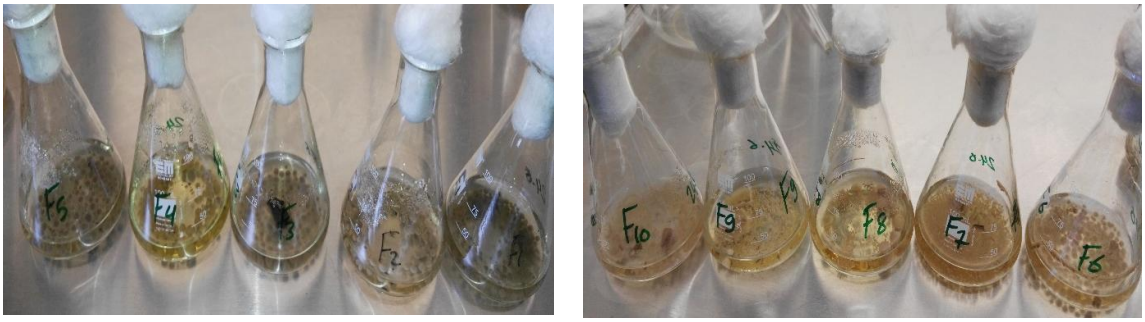


Fig 1: Quantitative pectinase assay of some fungal isolates

3.3. Molecular identification of fungal isolate, alignment in Genbank (Blast) and phylogenetic tree analysis

The nucleotide sequence of the NM-NRC isolate of ITS region sequence and was deposited in GenBank under the accession number OQ600201. In order to ascertain the probable identification of the strain, the acquired sequence was analyzed using BLAST queries using the "blastn" algorithm on the NCBI website (www.ncbi.nlm.nih.gov/BLAST). Based on sequence homology, the top hit from the BLAST analysis revealed that this nucleotide sequence exhibited 99% similarity with *Aspergillus niger*. Consequently, the isolate No. 1 was identified as *Aspergillus niger*. The phylogenetic tree constructed using the ITS region sequence demonstrated that the NM-NRC isolate belonged to the same group and was closely related to *Aspergillus niger* then named *Aspergillus niger* strain NM-NRC, **as depicted in fig 2.**

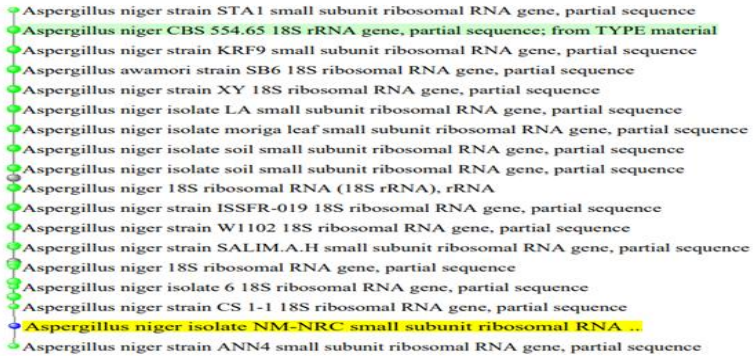


Fig. 2: Phylogenetic tree constructed based on ITS region sequences of *Aspergillus niger* NM-NRC with other *Aspergillus* species obtained from GenBank database

3.4. Aspergillus niger NM-NRC multistep mutation induction for pectinase production improvement

The NM-NRC strain underwent 60 minutes of ultraviolet (UV) radiation exposure during the first stage of mutation. The fifty colonies that managed to survive were then separated and their unique pectinase activity examined. Of these colonies, only three mutants showed any appreciable level of

efficacy concerning the particular activity of pectinase. The results showed that, with a protein content of 1.21±0.092 mg/ml, the mutant UV-28 had the highest degree of activity, measured at 76.32 U/mg. The natural form of the species, *Aspergillus niger* NM-NRC strain, had a protein content of 1.17±0.02 mg/ml and had a pectinase-specific activity of 51.47 U/mg.

Compared to the value that was previously indicated, this one is far lower. In the second step of the mutation process, the mutant UV-28 was exposed to 10 mg/ml of ethidium bromide (Eth.Br) for 60 minutes. Thirty colonies in all that had made it through the exposure were separated and closely inspected to determine whether they possessed any special pectinase-producing capacity. Only three of the mutants in the total dataset showed a notable degree of efficacy in pectinase-specific activity. The results showed that after 60 minutes of exposure to Eth.Br., the mutant E-9 showed the most hyperactivity compared to all other mutants. A specific value of 97.54 units per milligramme (U/mg) was shown for the activity, while 1.28±0.042 mg/ml of protein were present. By contrast, the protein concentration of 1.21±0.092 mg/ml and specific activity of 76.32 U/mg were demonstrated by the UV-28 wild type mutant.

In the third phase of the mutation process, 5 µl of a 30% solution was used as the concentration of H_2O_2 that was applied to the mutant E-9. Forty-five colonies in all that made it through the 60-minute exposure were divided up and analyzed to determine their pectinase-specific activity. Merely four mutants demonstrated a significant degree of effectiveness concerning pectinase-specific activity. Mutant H-34 was found to have the highest amount of hyperactivity, with a protein concentration of 1.34±0.020 mg/ml and a specific activity of 113.43 U/mg. By contrast, the protein content of the wild-type mutant E-9 was 1.28±0.042 mg/ml and its specific activity was 97.54 U/mg, **as shown in Table 2.**

Table 2: Estimation of pectinase specific activity produced by strain *Aspergillus niger* NM-NRC and its mutants after 3 days incubation at 30°C.

Strain	Pectinase specific activity (U/mg)	Protein content (mg/ml)
First step mutation with ultra violet (UV) mutagenesis		
Parent *Aspergillus niger* NM-NRC	**51.47**	**1.17±0.02**
UV mutants		
UV-13	74.80	1.18±0.054
UV-28	**76.32**	**1.21±0.092**
UV-42	64.75	1.12±0.012
Second step ethidium bromide (Eth.Br)* mutagenesis		
Parent UV-28	**76.32**	**1.21±0.092**
Eth.Br mutants		
E-9	**97.54**	**1.28±0.042**
E-24	90.70	1.26±0.060
E-29	88.66	1.23±0.041
Third step mutation ethidium bromide (H_2O_2) mutagenesis**		
Parent E-9	**97.54**	**1.28±0.042**
H_2O_2 mutants		
H-7	107.39	1.27±0.039
H-26	101.68	1.21±0.024
H-34	**113.43**	**1.34±0.020**
H-42	98.27	1.20±0.016

* EthBr 10 mg/ml concentration ** H_2O_2 5 µl of 30% concentration

3.5. Screening of significant carbon and nitrogen variables

Many carbon and nitrogen sources were investigated in order to identify the best ones for the mutant strain of *Aspergillus niger* NM-NRC H-34 to produce pectinase from. The findings revealed that the medium containing glucose (93.37 U/mg) and beef extract (96.48 U/mg) demonstrated the highest levels of pectinase activity, **as presented in fig 3a and 3b.**

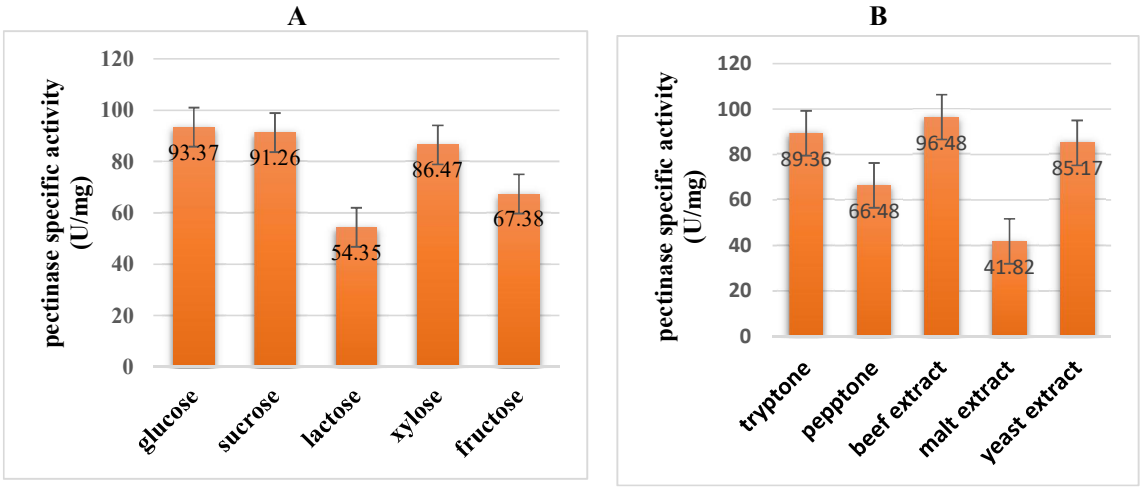


Fig 3: Medium optimization conditions by supplementing different A; carbon sources. B; nitrogen sources of mutant *Aspergillus niger* H-34.

3.6. Optimizing of culture conditions for pectinase activity through response surface methodology (RSM).

In order to enhance and optimize the yield of pectinase produced by a mutated strain of fungus, it is essential to assess the complex data presented using response surface methodology (RSM) for *Aspergillus niger* NM-NRC H-34. The work employed a central composite model to produce pectinase by conducting an 86-run experimental design on a mutant strain of *Aspergillus niger* NM-NRC H-34. The matrix comprised four components, with each component having three levels (-1, 0, and +1). Additionally, there were three replicas located at the center point. **Table 3**, the study presents the independent components using a coded matrix and provides the associated solutions, as well as the actual and expected values for pectinase activity. The variability in enzyme activity observed during the 86 iterations of the experiment can be attributed to the different settings used in each iteration. This highlights the importance of statistically optimizing fermentation conditions compared to traditional methods. The culture setting was optimized to achieve the highest pectinase activity by adjusting the temperature to 30ºC, pH to 9, incubation time to 48 hours, and using a citrus pectin concentration of 2%, along with 1.5% glucose and 1.5% beef extract as carbon and nitrogen sources, respectively. This optimization resulted in an activity of 164.45 U/mg (Run 52). The coefficient of determination (R^2) indicates a high level of precision in the model, with a value of 0.9934. The observed association is statistically significant, providing evidence that the current model for pectinase synthesis is reliable. The equation representing the final result, taking into account second-order elements, may be expressed as follows: $Y = -47.90 + 3.47X1 + 9.03X2 + 7.90X3 + 8.60X4 - 1.77X1X2 - 2.07X1X3 - 1.27X1X4 - 3.37X2X3 - 3.30X2X4 - 1.73X3X4 - 2.63X1^2 - 4.23X2^2 - 4.57X3^2 - 4.37X4^2$. Where Y represents the response or pectinase yield, and X1, X2, X3, and X4 are temperature, pH, incubation time, pectin concentration, glucose percentage and beef extract percentage.

Table 3: Design of different trials of the response surface methodology for independent variables and responses by mutant *Aspergillus* mutant H-34.

Run	A: Temperature	B: pH	C. carbon Source glucose	D. nitrogen Source beef extract	E: incubation time	F: pectin concentration	pectinase activity U/mg Actual value	Predicted value
1	35	9	0.5	0.5	72	3	162.41	162.77
2	30	7	1.5	1.5	72	2	145.34	145.39
3	30	7	1.5	1.5	48	2	143.28	144.57
4	35	9	0.5	2.5	72	3	161.39	162.33
5	35	9	2.5	2.5	72	1	162.83	162.72
6	35	5	0.5	0.5	72	1	124.33	125.25
7	25	5	2.5	2.5	72	3	125.53	125.74
8	25	9	0.5	0.5	24	1	162.46	162.25
9	25	9	2.5	0.5	72	3	163.33	162.80
10	25	9	2.5	2.5	24	1	163.22	163.64
11	30	7	1.5	1.5	48	2	144.34	144.57
12	25	5	2.5	2.5	24	1	123.39	125.39
13	35	9	0.5	2.5	72	1	163.12	161.88
14	35	5	2.5	2.5	24	3	122.24	122.47
15	30	7	1.5	1.5	48	2	142.23	144.57
16	25	5	2.5	0.5	24	1	126.66	124.83
17	35	9	0.5	0.5	24	3	163.55	162.41
18	35	9	2.5	0.5	24	1	160.02	162.37
19	25	9	2.5	0.5	24	3	161.49	161.90
20	30	7	1.5	1.5	48	2	143.31	144.57
21	35	7	1.5	1.5	48	2	146.37	145.08
22	35	9	2.5	0.5	72	1	163.46	162.54
23	25	5	0.5	0.5	72	1	125.77	124.59
24	35	5	0.5	0.5	72	3	124.95	125.23
25	30	7	1.5	1.5	48	2	143.11	144.57
26	25	9	2.5	2.5	72	3	161.32	163.42
27	35	5	2.5	0.5	72	1	125.48	125.78
28	25	5	2.5	2.5	72	1	126.63	126.25
29	35	9	0.5	0.5	72	1	163.44	162.11
30	25	5	0.5	2.5	24	1	124.44	124.55
31	30	7	1.5	1.5	48	2	145.55	144.57
32	35	5	2.5	2.5	72	3	126.38	124.77
33	25	7	1.5	1.5	48	2	144.68	145.28
34	35	9	2.5	0.5	72	3	162.51	162.75
35	30	7	1.5	1.5	48	2	144.39	144.57
36	35	9	0.5	2.5	24	1	162.72	162.82
37	35	5	2.5	0.5	24	1	125.53	124.35
38	25	5	2.5	2.5	24	3	123.41	123.80
39	25	9	2.5	2.5	24	3	163.71	162.73
40	30	7	0.5	1.5	48	2	144.33	143.46
41	30	7	1.5	1.5	48	2	146.63	144.57
42	25	5	0.5	0.5	24	3	121.12	123.48
43	35	5	2.5	2.5	72	1	124.42	125.46
44	35	9	2.5	0.5	24	3	161.11	161.50
45	30	5	1.5	1.5	48	2	126.74	126.30
46	25	9	2.5	0.5	24	1	161.49	162.59
47	35	9	2.5	2.5	24	1	162.66	162.77
48	25	9	2.5	2.5	72	1	163.24	163.23
49	35	9	2.5	2.5	72	3	163.59	162.72
50	30	7	2.5	1.5	48	2	143.69	143.87
51	25	5	0.5	2.5	24	3	123.37	123.41
52	30	9	1.5	1.5	48	2	164.45	164.20
53	35	5	0.5	2.5	72	3	124.77	124.30

54	35	5	0.5	2.5	24	1	126.45	124.21
55	35	9	0.5	2.5	24	3	160.64	162.18
56	30	7	1.5	1.5	48	3	145.56	145.52
57	35	9	2.5	2.5	24	3	162.35	161.68
58	30	7	1.5	0.5	48	2	142.31	141.38
59	35	5	0.5	2.5	72	1	122.44	124.53
60	25	9	2.5	0.5	72	1	163.55	162.39
61	30	7	1.5	1.5	48	2	142.77	144.57
62	25	5	2.5	0.5	24	3	124.36	123.46
63	25	5	0.5	2.5	72	1	125.37	124.53
64	30	7	1.5	1.5	48	1	146.63	145.98
65	35	5	0.5	0.5	24	1	124.41	124.71
66	25	9	0.5	0.5	72	3	161.65	162.02
67	25	5	2.5	0.5	72	3	125.59	125.62
68	25	5	0.5	0.5	24	1	123.47	124.40
69	25	5	0.5	0.5	72	3	124.37	124.75
70	25	9	0.5	2.5	24	3	162.88	162.44
71	35	5	0.5	2.5	24	3	121.13	122.88
72	25	5	0.5	2.5	72	3	126.62	124.47
73	25	9	0.5	0.5	24	3	163.47	162.01
74	35	5	2.5	2.5	24	1	124.49	124.25
75	30	7	1.5	1.5	24	2	145.52	144.78
76	30	7	1.5	1.5	48	2	146.64	144.57
77	35	5	2.5	0.5	24	3	122.27	122.79
78	25	9	0.5	2.5	72	3	162.52	162.24
79	30	7	1.5	2.5	48	2	141.21	141.44
80	25	9	0.5	2.5	72	1	160.34	161.60
81	35	5	0.5	0.5	24	3	125.41	123.60
82	35	9	0.5	0.5	24	1	161.99	162.83
83	25	9	0.5	0.5	72	1	160.06	161.17
84	35	5	2.5	0.5	72	3	124.49	125.30
85	25	5	2.5	0.5	72	1	125.33	125.91
86	25	9	0.5	2.5	24	1	162.94	162.90

3.7. The model validation

The validity of the proposed model was evaluated by predicting the pectinase production of the yeast mutant *Aspergillus niger* NM-NRC H-34 for each experiment in the matrix. The data acquired from the experiment, **as presented in Table 4**, demonstrate that the maximum observed pectinase production reached 164.45 U/ml, which is very near to the anticipated value of 164.20 in run 52. **Table 5** presents a comprehensive statistical analysis of the variance data about the synthesis of pectinase by the mutant H-34. The model had a strong level of statistical significance, as indicated by a F value of 478.35. This was further confirmed by Fisher's F test, which yielded a probability value (P model > F) of 0.01. The experiments exhibited enhanced precision and dependability as shown by 'Prob>F' values below 0.05 and a significantly decreased coefficient of variation. The results revealed a significant association between the observed and projected values, with all variables having a considerable impact on the pectinase production statistics. The statistical optimization in this study led to a significant enhancement in pectinase biosynthesis compared to the initial medium; reaching a level of 164.45 U/mg. **Figure 4** demonstrates the influence of multiple factors and their interconnectedness on the synthesis of pectinase. Response surface curves were generated to depict the interaction among multiple factors and determine the ideal quantity of each variable that yields the maximum response. Each picture illustrates the effect of two parameters while holding all other factors at a constant value of zero. The highest reaction value was recorded at a temperature of 30°C, pH level of 9, after 48 hours of incubation. The reaction was conducted using a citrus pectin concentration of 2%, while 1.5% glucose and 1.5% beef extract were used as carbon and nitrogen sources, respectively.

Table 4: Analysis of variance (ANOVA) for Response Surface Quadratic Model CCD) by *Aspergillus* mutant H-34.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	23774.88	27	880.55	478.35	< 0.0001	Significant
A-temp	0.6264	1	0.6264	0.3403	0.5619	
B-pH	23695.08	1	23695.08	12871.98	< 0.0001	
C-C. Source glucose	2.74	1	2.74	1.49	0.2277	
D-N. Source beef extract	0.0559	1	0.0559	0.0303	0.8623	
E-incubation time	6.18	1	6.18	3.36	0.0720	
F-pectin concentration	3.54	1	3.54	1.92	0.1707	
AB	0.2970	1	0.2970	0.1614	0.6894	
AC	2.53	1	2.53	1.37	0.2460	
AD	1.72	1	1.72	0.9358	0.3374	
AE	0.5077	1	0.5077	0.2758	0.6015	
AF	0.1388	1	0.1388	0.0754	0.7846	
BC	0.0342	1	0.0342	0.0186	0.8920	
BD	0.9851	1	0.9851	0.5351	0.4674	
BE	6.41	1	6.41	3.48	0.0670	
BF	1.88	1	1.88	1.02	0.3159	
CD	0.6521	1	0.6521	0.3542	0.5540	
CE	3.14	1	3.14	1.71	0.1966	
CF	0.8145	1	0.8145	0.4425	0.5086	
DE	0.1849	1	0.1849	0.1004	0.7524	
DF	0.1892	1	0.1892	0.1028	0.7497	
EF	4.75	1	4.75	2.58	0.1135	
A^2	0.8812	1	0.8812	0.4787	0.4918	
B^2	1.10	1	1.10	0.5954	0.4435	
C^2	1.97	1	1.97	1.07	0.3055	
D^2	23.81	1	23.81	12.94	0.0007	
E^2	0.6271	1	0.6271	0.3407	0.5617	
F^2	3.31	1	3.31	1.80	0.1851	
Residual	106.77	58	1.84			
Lack of Fit	84.29	49	1.72	0.6886	0.8074	not significant
Pure Error	22.48	9	2.50			
Cor Total	23881.65	85				

Factor coding is **Coded**.
Sum of squares is **Type III – Partial**

The Model F-value of 478.35 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 0.69 implies the Lack of Fit is not significant relative to the pure error. There is a 80.74% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

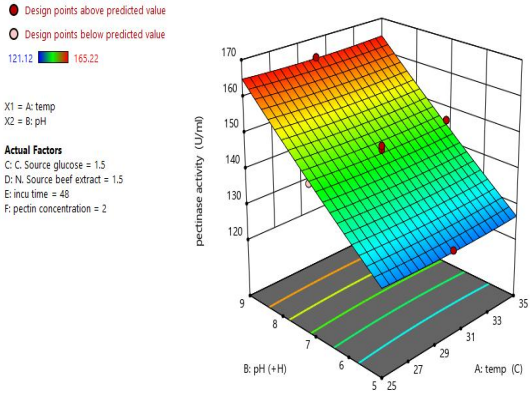
Table 5: Fit statistics

Std. Dev.	**1.36**	**R^2**	**0.9955**
Mean	143.73	**Adjusted R^2**	0.9934
C.V. %	0.9439	**Predicted R^2**	0.9906
		Adeq Precision	53.9003

The Predicted R^2 of 0.9906 is in reasonable agreement with the Adjusted R^2 of 0.9934; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 53.900 indicates an adequate signal. This model can be used to navigate the design space.

A



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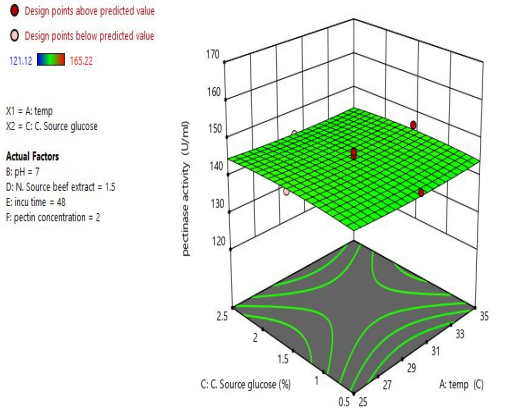

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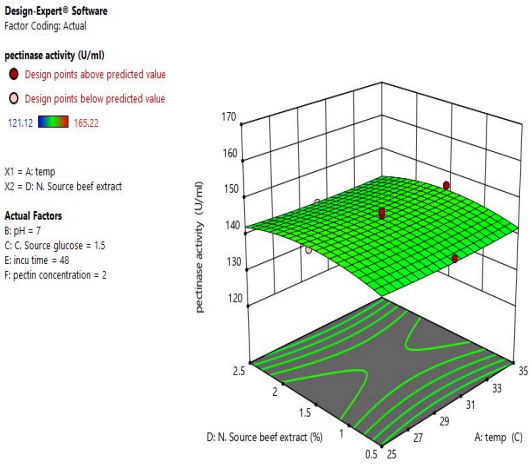

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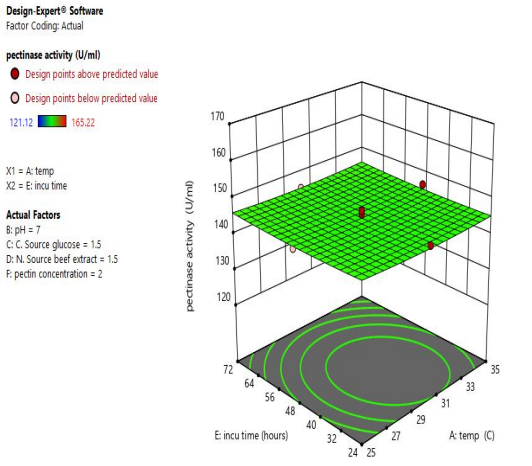

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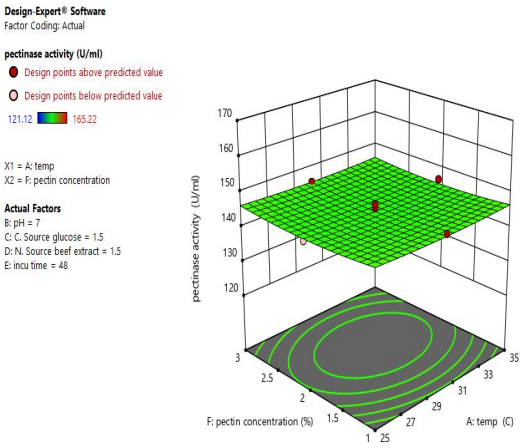

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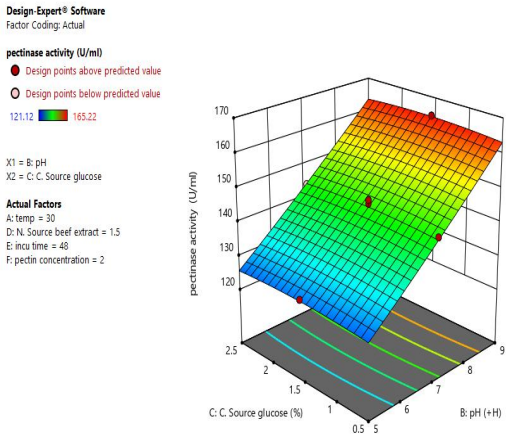

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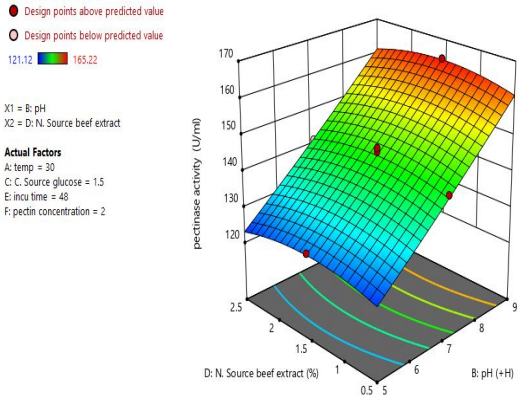
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Design points below predicted value
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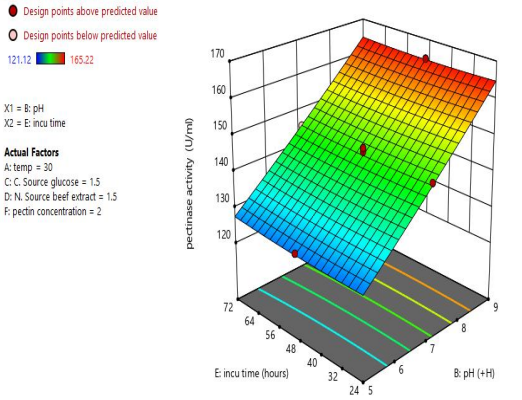
165.22
X1 = B: pH
X2 = D: N. Source beef extract
Actual Factors
A: temp = 30
C: C. Source glucose = 1.5
E: incu time = 48
F: pectin concentration = 2
pectinase activity (U/ml)
D: N. Source beef extract (%)
B: pH (+H)

H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Actual Factors
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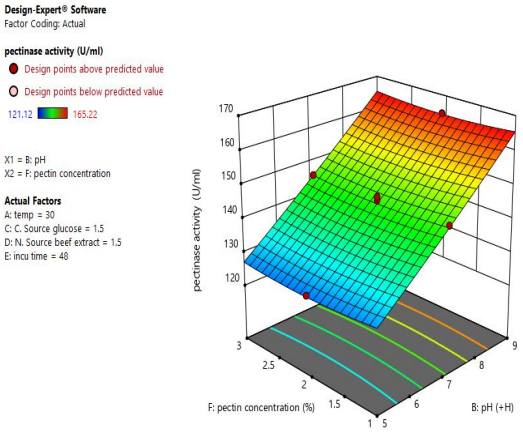
B: pH (+H)

I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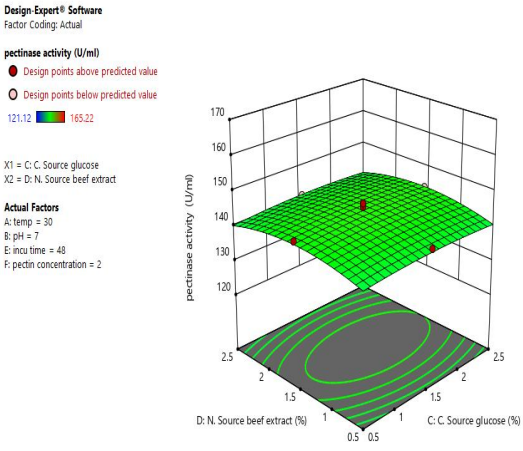
F: pectin concentration (%)
B: pH (+H)

J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Actual Factors
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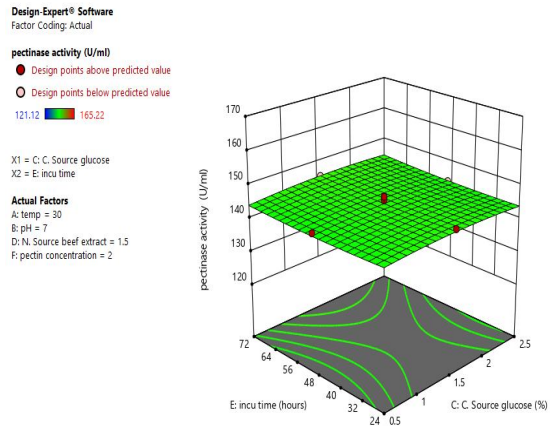
F: pectin concentration = 2
pectinase activity (U/ml)
D: N. Source beef extract (%)
C: C. Source glucose (%)

K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X2 = E: incu time
Actual Factors
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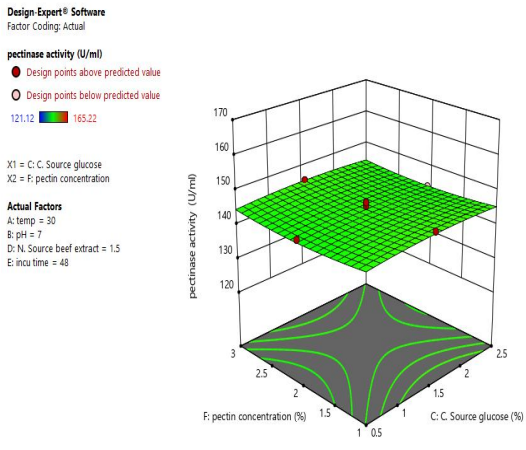
B: pH = 7
D: N. Source beef extract = 1.5
F: pectin concentration = 2
pectinase activity (U/ml)
E: incu time (hours)
C: C. Source glucose (%)

L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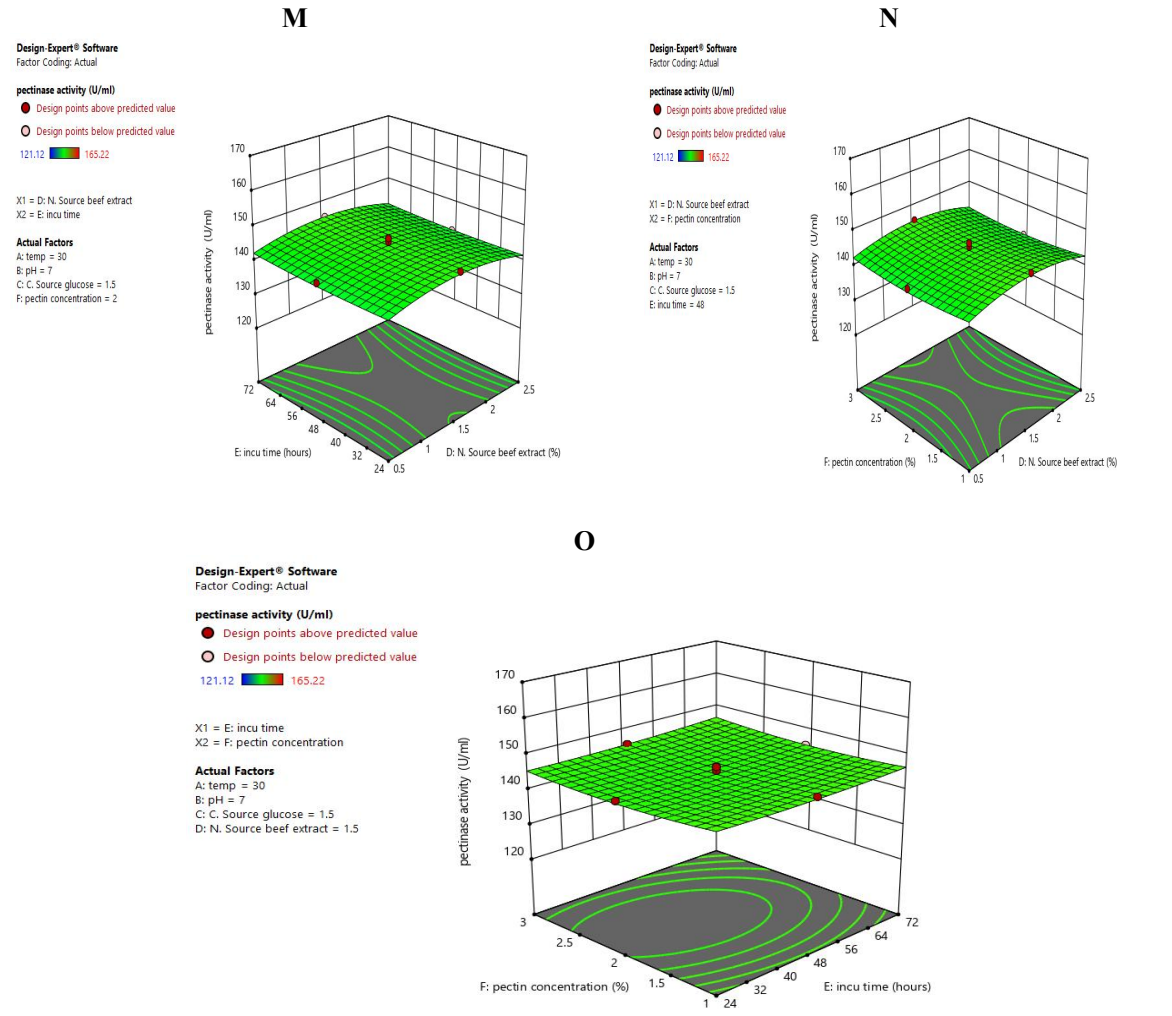

Fig 4: Contour plots of pectinase activity as a function of the interactions of four variables by keeping the other at centre level. **(a)** temp., pH **(b)** glucose, temp. **(c)** beef extact, temp. **(d)** temp., incub.time **(e)** pectin conc., temp. **(f)** glucose, pH **(G)** beef extract and pH **(H)** incub.time and pH **(I)** pectin conc. and pH **(J)** beef extract and glucose **(K)** incub.time and glucose **(L)** pectin conc. and glucose **(M)** incub.time and beef extact **(N)** pectin conc. and beef extact and **(O)** pectin conc. and incub.time, on pectinase production by mutant H-34.

4. Discussion

Fifteen fungal isolates were collected from ten soil samples, and then screened and assessed for their pectinase activity. Among these isolate, isolate No. 1 which identified as *Aspergillus niger* NM-NRC, which was isolated from Cairo, Egypt, produced the highest amount of extracellular pectinase (51.47 U/mg), after three days incubation. KC *et al.* (2020), 1% citrus pectin integrated potato dextrose agar (PDA) was used as the primary screening medium for 55 fungal strains that were isolated from 20 different soil samples. Pectinase screening agar medium (PSAM) was used as a secondary screening media for pectinolytic organisms. Of them, *Aspergillus spp*. Gm produced the most enzymes when cultured for 48 hours at 1% substrate concentration and 30°C. (Lara-Márquez *et al.,* 2011) the isolation of pectinase-producing fungal is specifically identified as *Colletotrichum gloeosporioides*. According to the paper (Sahay *et al.,* 2013) the study recorded the identification of two strains of yeast, *Rhodotorula mucilaginosa* PT1 and *Cystofilobasidium capitatum* SPY11, which exhibited the capacity to degrade pectin. These yeasts demonstrated the ability to operate at temperatures lower than their ideal range, exhibiting 50-80% of their maximum activity. In addition, they flourished in oenological environments with pH levels ranging from 3 to 5. (Haile and Kang, 2019) the identification of 28 yeast species was

made; these included *Saccharomyces cerevisiae*, *Papiliotrema flavescens*, *Saccharomyces anomalus*, and *Pichia kudriavzevii*, all of which are capable of producing pectinase enzymes. With rises of 178%, 160%, and 152%, respectively, the pectin degradation index of *S. fibuligera, W. anomalus*, and *P. flavescens* was much higher than that of the other species. The outcomes were consistent with earlier research suggesting that *PG* is the principal enzyme generated by pectinolytic yeasts (Combo *et al.,* 2012).

Mutation is recognized as a process that alters the DNA sequence of a specific gene, and mutagenesis is the source of genetic differences (Akbar *et al.,* 2013). Multiple studies have been conducted to improve the production of pectinase by employing gene editing and mutation techniques. When employing a conventional genetic technique for mutagenesis, random alterations are utilized to augment the production of desired metabolites in a natural strain. Although the DNA recombinant technique has been highly beneficial in the creation of commercial enzymes, random mutagenesis continues to be the preferred and widely employed method for generating consistent strains (Xin, 2012). The advantages of employing chemical mutagens for random mutagenesis are substantial, mainly because to their simplicity and cost-effectiveness when compared to recombinant DNA methods. The occurrence of stochastic mutation has garnered significant interest because of its capacity to enhance bacterial enzyme output. Common physical mutagenesis techniques involve the use of gamma rays, X-rays, and ultraviolet radiation. Chemical mutagenesis approaches involve the use of colchicine, hydrogen peroxide , ethyl methane sulfonate, sodium azide , and ethidium bromide (Akbar *et al.*, 2015). These compounds have been recognized as powerful mutagens that increase the synthesis of pectinase in strains capable of producing it (Lateef *et al.,* 2015).

This paper outlines the utilization of three efficient mutagenesis methods, namely ultraviolet, hydrogen peroxide, and ethidium bromide, to alter the original wild-type strain *Aspergillus niger* NM-NRC. The aforementioned techniques were utilized in a sequential mutation induction procedure to augment the production of pectinase. As a result, several different mutant cultures were produced, including one called mutant *Aspergillus niger* H-34. This mutant strain had a pectinase activity of 113.43 U/mg, over the wild type *Aspergillus niger* NM-NRC had a pectinase activity of 51.47 U/mg. The current study demonstrated the advantageous effects of employing ultraviolet, hydrogen peroxide, and ethidium bromide for mutagenesis in order to enhance pectinase output. The direct method resulted in strains that showed increased enzyme synthesis compared to the original wild type strain. (Heerd *et al*., 2014), the researchers found that, following exposure to physical and chemical mutagens, the *polygalacturonase* output of the strains of fungi that were studied was 2.4 times more than that of the wild-type strains. (Muzzamal and Latif, 2016) prior research has demonstrated that the utilization of induced UV and acridine orange mutagenesis resulted in an enhanced production of *polygalacturonase* in *Bacillus* and *Aspergillus tamari* strains. *Bacillus subtilis* and *Bacillus amyloliquefaciens* isolates were also subjected to mutation. The results demonstrated that the mutants exhibited a threefold increase in *polygalacturonas*e enzyme production compared to the original strains. Yin *et al.* (2016) the study found that the BM-201 strain of *Fusarium oxysporum*, when exposed to ultraviolet treatment, had a pectinase activity that was 73.6% higher than that of the original strain. (Akbar *et al.,* 2015). *Aspergillus carbonarius* underwent alterations as a result of exposure to hydrogen peroxide, ultraviolet light, colchicine, and ethidium bromide. Pectinase activity was greatest in the mutant E8, outperforming the natural type by a ratio of 1.8.

The primary goal of this study was to determine the ideal circumstances for pectinase synthesis by assessing the effects of several factors. Using this method, we were able to significantly improve the environmental factors required in order to produce enzymes at their best. Using response surface approach, we looked into the primary and interacting effects of multiple environmental conditions on pectinase synthesis at the same time. The results showed that the mutant strain *Aspergillus niger* NM-NRC H-34 exhibited the greatest level of pectinolytic activity when incubated at a temperature of 30^{o}C, a pH of 9, for a duration of 48 hours, and with a pectin concentration of 2%, and carbon and nitrogen sources consisting of 1.5% glucose and 1.5% beef extract, respectively. Under these conditions, the strain produced a pectinolytic activity of 164.45 U/mg. These results support previous research that has also utilized Response Surface Methodology to enhance enzyme production under various culture conditions, (Ire *et al.*, 2018) Utilized Response Surface Methodology to enhance the efficiency of invertase production from *Aspergillus niger* cultivated on inexpensive agricultural residues. George *et al.* (2021), from the results obtained, the optimum conditions using the Box-Behnken design for *Aspergillus*

niger pectinase production were approximately; pH of 3.90; 5.87 days of fermentation at 21.24ºC; inoculum volume of 1.00 ml; particle size of 0.06-inch and 11.43 minutes agitation time. (KC *et al.,* 2020) the optimal conditions for the activity of the enzyme produced by *Aspergillus spp*. were found to be a temperature of 30 ◦C (with an activity of 75.4 U/mL), a pH of 5.8 (with an activity of 72.3 U/mL), and a substrate concentration of 0.5% (with an activity of 112.0 U/mL).

5. Conclusion

The present work involved the evaluation of multiple fungal strains for their ability to produce pectinase. *Aspergillus niger* NM-NRC exhibited the most significant synthesis of the pectinase enzyme. The expression of pectinase in *Aspergillus niger* NM-NRC was enhanced by using physical ultraviolet and chemical mutagenesis; as well ultraviolet, hydrogen peroxide and ethidium bromide. The mutant strain, *Aspergillus niger* H-34, demonstrated higher levels of pectinase activity of 113.43 U/mg, in comparison to its wild-type counterpart *Aspergillus niger* NM-NRC 51.47 U/mg. The enhancement of microbial enzyme production by mutant H-34 was accomplished by the utilization of response surface approaches, resulting in a synergistic amalgamation of efficient parameter interactions. The most favorable parameters for the production of pectinase enzyme were determined to be a temperature of 30ºC, pH level of 9, an incubation period of 48 hours, a pectin concentration of 2%, and the use of 1.5% glucose and 1.5% beef extract as carbon and nitrogen sources, respectively.

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Author Contributions:

Both authors designed and carried out the experimental procedures and research methodology. Participated in the writing, editing, and revision of articles in addition to data analysis and illustration. Prepared the figures and read the manuscript's corresponding parts.

Funding:

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Data Availability

The sequenced identification of *Aspergillus niger* NM-NRC which was deposited in the NCBI database under the accession number OQ600201. All the remaining data supporting the findings of this study are available within the article.

Declarations

Ethics approval

Not applicable.

Consent to publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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