Optimization and comparative studies on activities of β-mannanase from newly isolated fungal and its mutant

Om K.H. Khattab[a], Siham A. Ismail[b], Nivien A. Abosereh[c], Amany A. Abo-Elnasr[a], Shaimaa A. Nour[b], Amal M. Hashem[b]

[a]Department of Plant and Microbiology, Science Collage, Helwan University, Helwan, [b]Department of Chemistry of Natural and Microbial Products, Pharmaceutical and Drug Industries Research Division, Nationa Research Centre, Giza, [c]Department of Microbial Genetics, Division of Genetic Engineering and Biotechnology, Nationa Research Centre, Giza, Egypt

Correspondence to Amal Mohamed Hashem, Professor in Department of Chemistry of Natural and Microbial Products, Pharmaceutical and Drug Industries Research Division, National Research Centre, El Buhouth St., Dokki, Giza, Egypt. Tel: +2 01062559809-fax: (+202) 33370931; e-mail: amal_mhashem@yahoo.com

Received: 25 August 2019
Revised: 2 October 2019
Accepted: 8 October 2019
Published: 24 March 2020

Egyptian Pharmaceutical Journal 2020, 19:29–46

Background and objective
β-Mannanase has potential industrial applications in pharmaceutical field, bioethanol production, coffee extraction, food and feed technology, etc. So finding a new and promising enzyme source is a very important issue. The aim of this study was to improve the biosynthesis of β-mannanase by different techniques, such as mutation and optimization of the culture parameters.
Materials and methods
Five fungal isolates were tested for the production of β-mannanase. Enzyme activity, protein content, and specific activity were determined. The most potent isolated microorganism and its mutant were identified by using Transmission Electron Microscopy and 18SrDNA sequencing and phylogenetic analysis. Ultraviolet and gamma rays were used. Optimization studies were done to maximize enzyme production from the most potent microorganism and its highly productive and stable mutant, including culture conditions and medium compositions, and statistical optimization was also carried out. Primary characterization of β-mannanase was studied.
Results and conclusion
In our research, we found a stable mutant strain obtained by using gamma radiation at 150 GY. The first step of the fermentation, optimized by one-factor-at-a-time technique, increased the biosynthesis of β-mannanase for *Penicillium citrinium* 150 GY from 65.9 to 219 IU/ml compared with the wild strain, which increased from 16.82 to 26.5 IU/ml.
Statistical optimization improved *P. citrinium* 150 GY β-mannanase from 219 to 296 IU/ml by applying Plackett–Burman design and increased the level of β-mannanase biosynthesis to 351 IU/ml. Primary characterization of β-mannanase produced by *P. citrinium* and *P. citrinium* 150 GY proved that they are almost the same, except in a little shift to higher value (5°C) in optimum temperature.

Keywords:
gamma radiation, *Penicillium citrinium* 150 GY, *Penicillium citrinium*, β-mannanase

Egypt Pharmaceut J 19:29–46

1687-4315

Introduction

β-Mannanase is the enzyme that cleaves the β-1,4-mannosidic linkages in mannans, galactomannans, glucomannans, and galactoglucomannans, which are found in many natural products (Fig. 1) [2,3].

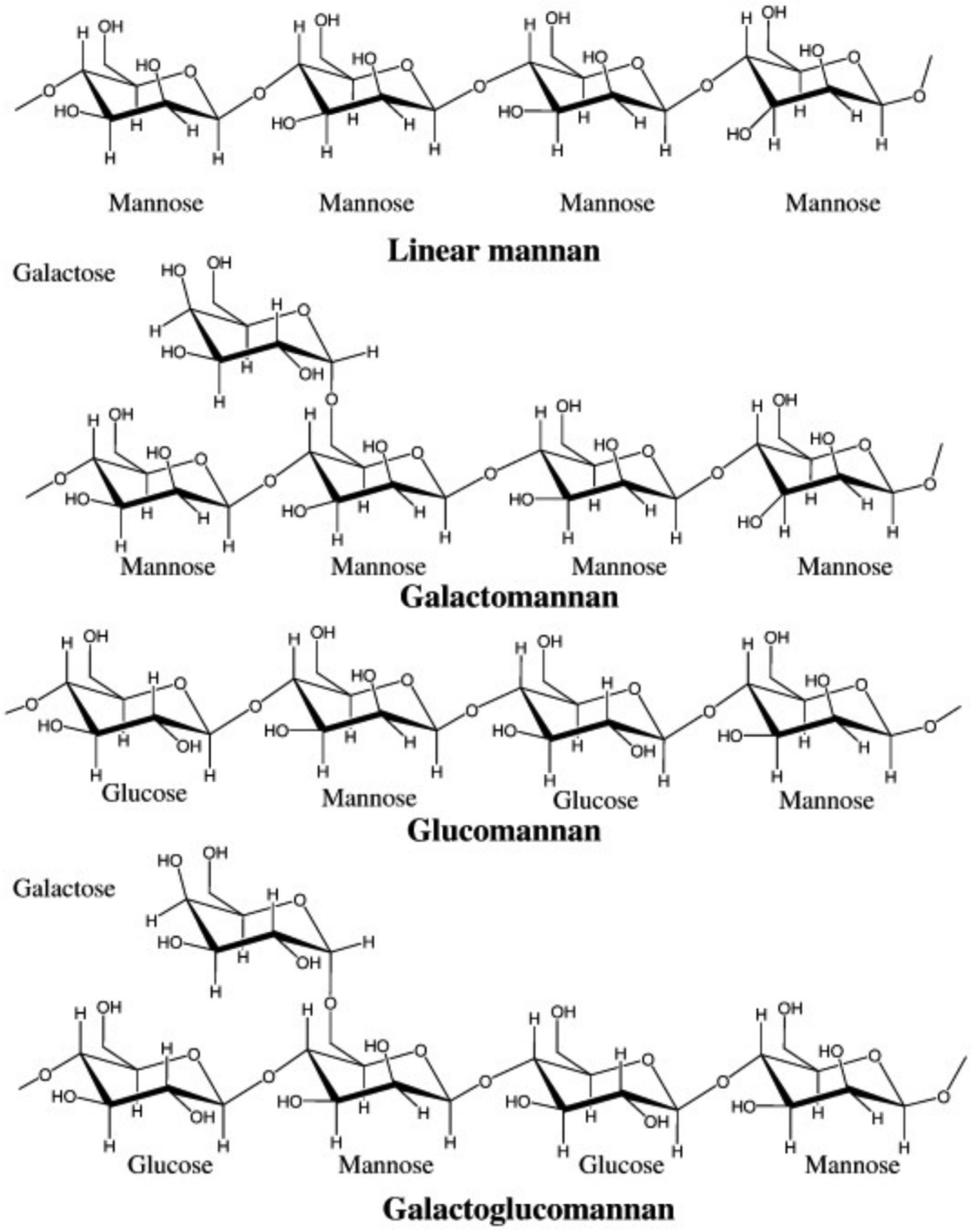
β-Mannanase is produced by different microorganisms [4,5]. The fungal strains are preferred owing to high yields and extracellular release of the enzymes and higher enzymatic activity [6,7].

Owing to extensive use of β-mannanase in different industries, the interest in achieving microbial strains with high β-mannanase activity has increased. Therefore, several methods were used to increase β-mannanase biosynthesis, such as optimization of enzyme production, cloning of β-mannanase gene, random mutagenesis, and site-specific mutagenesis [8].

Random mutagenesis is often used as a rapid and feasible method. This technique relies on the exposure of the microorganism to a physical or a chemical mutagen. Physical mutagenesis can be performed with electromagnetic radiation, such as ultraviolet (UV) light, radiographies, gamma rays, or a particle radiation with beta and alpha particles or fast and thermal neutrons [7,9].

Chemical mutagenesis can be achieved with alkylating agents (*N*-methyl-*N*'-nitro-nitrosoguanidine, ethyl

Figure 1



Structural chemistry of mannan, galactomannan, glucomannan, and galactoglucomannan, taken from Srivastava *et al.* [1].

methane sulfonate, *N*-methyl-*N*-nitrosourea, dimethyl nitrosamine, etc.), intercalating agents (ethidium bromide), or azides (sodium azide) [10,11].

The current research was carried to find out new fungal isolates that can produce a significant level of β-mannanase using a cheap substrate, coffee waste.

Morphological characterization and phylogenic analysis were applied to identify the chosen fungal isolate. Mutagenesis of the most potent fungal strain was

achieved by gamma radiation to enhance the production of β-mannanase.

Furthermore, optimization of the production of β-mannanase was performed by one-factor-at-a-time technique for both wild and mutant strains. The statistical methodology was applied to maximize the enzyme production of the most potent strain (wild or mutant). Primary characterization of the produced β-mannanase was also determined.

Materials and methods

Chemicals

Locust bean gum was obtained from Sigma Chemicals (St Louis, USA) (a galactomannan from the seeds of *Ceratonia siliqua*, which consists of a straight-chain polymer of mannose with galactose branches on every fourth mannose). There is No animal or human experimental. All other chemicals used were of analytical grade.

Coffee residue

Green coffee beans were bought from a local market, Giza, Egypt. They were roasted, and the spent coffee ground was then milled by thermal water extraction. The residue of coffee was collected and used in the microbial culture after determination of the carbohydrate contents [12].

Microorganisms

In this study, five fungal strains, primary identified as *Aspergillus niger.1*, *Aspergillus niger.2*, *Penicillium* spp. (isolated from Pharaoh mummies of Ancient Egyptian Museum, Cairo, Egypt), *Fusarium* spp., and *Rhizoctinia* spp. (isolated from plant), were screened for β-mannanase production.

All the fungal strains were maintained on potato dextrose agar (PDA) and incubated at 30°C for 7 days and stored at 4°C with monthly subculturing.

Mutagenesis with ultraviolet

UV mutagenesis to the spore suspension of *Penicillium citrinium* was done by treating with UV (power: 30 W and wavelength: 254 nm) each 15-min interval, with a distance of 18 cm from the UV light source, and then plates were stored in the dark for 2 h. Mutagenic suspension of each treatment was diluted and plated on PDA as complete media and Cazpek media as minimal media at 30°C for 5 days. Single colony with different morphology from each treatment was subcultured and tested for enzyme production [13].

Mutagenesis with gamma rays

In this way, mutagenesis of the spore of the most potent isolate was carried out using five different doses of gamma radiation (0, 50, 100, 150, 200, and 250 Gray) produced from Cobalt-60 (Co60) (Egyptian Atomic Energy Authority, Nasser, Egypt) [14].

Strain identification, 18s rDNA sequencing, and phylogenetic analysis

The most potent isolated microorganism and its mutant were identified by using Transmission Electron Microscopy (TEM, JEM-2100; JEOL, USA) after growing on PDA plate for 7 days. Molecular identification was carried out by using Easy Quick DNA extraction kit (Quiagene, Netherlands and Germany) following the manufacturer's instructions.

The PCR was performed according to Plengvidhya *et al.* [15], in a total of 25 µl reaction volume, and amplification was programmed to 40 cycles after an initial denaturation cycle for 2 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 25°C for 1 min, and an extension step at 72°C for 2 min, followed by extension for 10 min at 72°C in the final cycle.

Culture media

(1) Inoculum medium: this included the following (g/l): peptone, 2; ammonium sulfate, 1.5; urea, 0.3; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 10; and locust bean gum, 10 [16]. The pH of the medium was adjusted at 5.3 before autoclaving. Each 250 ml Erlenmeyer flask contained 50 ml of the medium and was autoclaved for 16 min at 121°C.
(2) Fermentation medium: the fermentation medium contains the following (g/l): peptone, 2.27; ammonium sulfate, 1.7; urea, 0.34; $MgSO_4 \cdot 0.7H_2O$, 0.6; K_2HPO_4, 7.5; and coffee waste (3 g/flask). The pH of the medium was adjusted at 4.5 before autoclaving. Each 250 ml Erlenmeyer flask contained 50 ml of the medium and was autoclaved for 16 min at 121°C [16].
(3) Cultivation conditions: an inoculum culture was obtained by culturing the fungal strains in the aforementioned medium at 30°C for 48 h with shaking at 120 rpm. The culture flasks were inoculated by 8% of the inoculum and incubated at 30°C in static condition and in a shaking incubator at 120 rpm for shaking of all the tested fungal species. Thereafter, the fermented medium was centrifuged, and the filtrate was used as the crude enzyme solution.

Enzyme assay

An assay was performed by incubating 0.5 ml of appropriately diluted culture filtrate with 1 ml of 1% (w/v) locust bean gum in sodium citrate buffer (50 mmol/l) at pH 5.5 for 10 min at 50°C [17]. The reducing sugars produced were determined using the Nelson–Somogyi technique [18]. One unit of enzyme activity was defined as the amount of enzyme that released 1 mmol of mannose/ml/min.

Protein determination

To determine the specific enzymatic activity, the quantification of total amount of soluble protein was measured using the Lowry et al. [19] method.

Optimization of β-mannanase production

One-factor-at-a-time technique

The ability of the wild and mutant strains to utilize coffee waste in the fermentation medium was tested at different incubation periods (3, 5, 7, 10, 12, 14, and 18 days), while testing with static and shaking (120 rpm) culture techniques. Effects of different nitrogen sources were investigated by replacing different equivalent amount of nitrogen base from different tested organic or inorganic nitrogen sources. Different organic nitrogen sources such as peptone, yeast extract, Baker's yeast, casein, corn steep liquor, and urea, whereas inorganic nitrogen sources, including sodium nitrate and ammonium sulfates, were individually tested as a sole nitrogen source.

The optimum concentration of the coffee waste and nitrogen source were evaluated in relation to enzyme yield. The strain that produced the highest β-mannanase was subjected to further optimization. The experiments were conducted in triplicate, and the results are the average of these three independent values.

Statistical optimization

(1) Plackett–Burman experimental design (PBD): to evaluate the relative important factors in the production of β-mannanase by the chosen isolate in substrate fermentation, PBD was applied [20]. Matrix contains seven dependent variables in two levels [(+1) and (−1)] with nine trials being selected, as described in Table 1. PBD was based on the first-order linear model:

$$Y = B0 + \Sigma BiXi \qquad (1)$$

(2) where Y is the response (β-mannanase production), $B0$ is the model intercept, and Bi is the linear coefficient and Xi is the level of the independent variable. The main effect of each variable was determined by following equation:

$$E(Xi) = 2(\Sigma Mi + -Mi-)/N \qquad (2)$$

where $E(Xi)$ was the effect of the tested variable Mi + and Mi– represented β-mannanase production from the trials where the independent variable (Xi) measured was present at high and low levels, respectively and N is the number of trials. The significance level (P value) of each tested variable was determined using Student's t-test.

(3) Optimization of factors using response surface methodology (RSM): RSM was employed to investigate the accurate optimal levels of the main affecting factors obtained from PBD using central composite design (Box and Behnken) [21].

A study of four variables at five different levels (−1, +1, 0, −2, and +2) was carried out in a set of 30 trials (Table 5).

A second-order polynomial equation was used for the interpretation of correlation between variables and the response.

The equation is presented as follows:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_i {X_i}^2 + \sum_{ij} \beta_{ij} X_i X_j \qquad (3)$$

Table 1 Survey of some fungal strains for the production of extracellular β-mannanase in shaken cultures

Microorganism	Time (days)	Enzyme activity (IU/ml)	Specific activity
Penicillium citrinium	5	3.29±0.66	0.48
	7	5.48±0.69	0.80
	10	10.43±3.79	1.45
	12	16.35±0.79	2.18
Aspergillus niger.1	5	0.661±0.21	0.09
	7	0.726±0.09	0.10
	10	0.643±0.07	0.09
	12	0.636±0.01	0.09
Aspergillus niger.2	5	0.317±0.09	0.04
	7	0.324±0.03	0.04
	10	0.298±0.07	0.04
	12	0.22±0.40	0.03
Fusarium spp.	5	0.744±0.13	0.12
	7	0.951±0.37	0.15
	10	1.02±0.19	0.15
	12	0.951±0.29	0.14
Rhizoctinia spp.	5	0.212±0.09	0.03
	7	0.212±0.07	0.03
	10	0±0.13	0
	12	0±0.06	0

where *Y* represents response or β-mannanase yield; $\beta 0$, is model interception; βi, is linear coefficient; βii, is squared coefficient; βij, is interaction coefficient; and *Xi* and *Xj* are the coded levels of independent variables.

Primary characteristics of the crude β-mannanase produced by wild and mutant strains

Effect of pH

The optimum pH of the crude β-mannanase for wild and mutant strain was determined by carrying out the enzyme assay at different pHs (4.0–7.0) using sodium citrate buffer (50 mmol/l) for 10 min at 50°C.

Effect of temperature

The optimum temperature of the crude β-mannanase of wild and mutant strains was investigated by incubating the reaction mixture at different temperature ranged from 40 to 65°C for 10 min under standard assay condition.

Determination of thermal stability

Thermal stability of the crude β-mannanase for wild and mutant was investigated by incubating the enzyme in sodium citrate buffer (50 mmol/l) solution at pH 5.5 (without substrate) at different temperatures (40–65°C) for different time intervals (15, 30, 45, 60, 90, and 120 min). The residual enzyme activity was measured under the optimum conditions.

All the experiments were done in triplicate, and the recorded data were the mean values.

Results and discussion

The production of extracellular β-mannanase from the fungal isolates

The shaking cultures of the five fungal isolates possessed different levels of β-mannanase, ranged from 0.0 to 16.3 IU/ml (Table 1). *Rhizocotinia* spp. failed to produce β-mannanase in all tested incubation periods, whereas *Penicillium* spp. recorded the highest β-mannanase activity (16.35 IU/ml) after 12 days and was chosen for further studies.

Microorganism Identification

The 18S rDNA gene sequence analysis indicated that the isolate is closely related to *P. citrinium* (Fig. 2), which was known as β-mannanase producer [22]. The data of 18 Sr DNA partial sequence have been submitted to Gen Bank databases under the name of *P. citrinium* with accession no. of Egy5 LC368457.

Mutation by using ultraviolet rays

Of all the mutants generated by this method, mutant designated UV30-B increased mannanase biosynthesis with ~threefold (48.34 IU/ml) higher than the wild type (16.48 IU/ml), whereas the lowest amount (0.79 IU/ml) was produced by mutant coded UV15-H. The mutant strains 30-B showed low stability within the three generation (unpublished data).

Mutation by using gamma rays

In an attempt to increase the production of β-mannanase, *P. citrinium* was mutagenized by exposing to different doses (50, 100, 150, 200, and 250 Gy).

The production of extracellular *P. citrinium* β-mannanase increased about 1.87 fold with mutant at dose 50 Gy (30.58 IU/ml), whereas at dose of 150 Gy, the resulted mutant increased the production (37 IU/ml) by about 2.27-fold more than the wild-type strain. To our knowledge, no one improved β-mannanase production by exposing the fungal strain to gamma rays. Fig. 3 shows high morphological difference between the wild strain (A&B) and the mutant (C&D) by using transmission electron microscopy.

Increasing enzyme production by exposure to the gamma radiation was recorded by other researches. Iftikhar *et al.* [23] found that the dosage 140 Gy in MBL-5 showed maximum extracellular lipases production. Moreover, Shahbazi *et al.* [14] recorded that gamma radiation at 250 Gy had accrued the activity of Ccase, CMCase, Avicellase, and Fpase for *Trichoderma* spp.

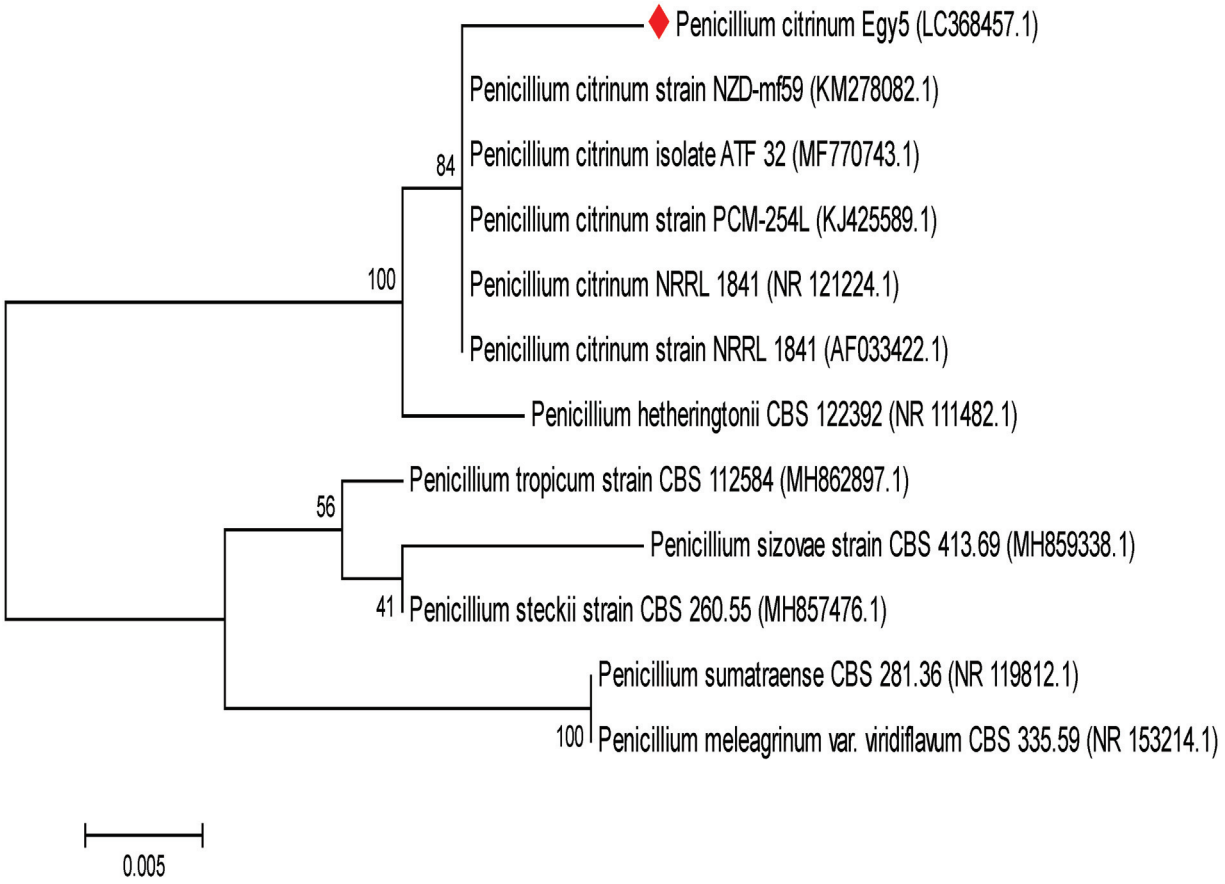
Optimization the production of *Penicillium citrinium* β-mannanase

In this study, we optimized the production of β-mannanase by *P. citrinium* and its 150-Gy mutant.

Effect of incubation period and culture technique on biosynthesis β-mannanase

The results in Fig. 4a, b illustrate that in shaking cultivation the highest amount of β-mannanase (65.9 IU/ml) was produced by 150 Gy strain followed by 28.16 IU/ml obtained by 50 Gy mutant strain after 18 days. The highest value obtained by the wild strain (16.82 IU/ml) was produced after 14 days in shaking culture. By using static culture technique, the produced β-mannanase was reduced to half of that produced by shaking cultivation for both 150 Gy mutant strain and 50 Gy mutant strain (34.5 and 16.28 IU/ml, respectively). The wild-type strain produced 5.4 IU/ml only with the static cultivation. Therefore, 150 Gy mutant strain was chosen with the wild-type strain (for comparison) for further experiments with shaking culture technique.

Figure 2



Molecular phylogenetic tree of *Penicillium citrinium* strain Egy5 with related strains. Phylogenetic analysis was conducted in MEGA6 software (Tamura *et al*., 2013) using Maximum Likelihood method and basing on genes of ribosomal RNA (rRNA) and internal transcribed spacer. Accession number is indicated adjacent to each strain between parentheses. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. Bootstrap values are indicated at nodes.

Effect of different concentration of coffee waste

Agroindustrial byproducts are available in large amounts, and they represent a cheap source for the production of several enzymes [16,24].

Since coffee waste is the byproduct produced in huge amount in the coffee industries, its uses as a carbon source in the fermentation medium can reduce the cost of the enzymatic synthesis and also solve the pollution problems.

The effect of different concentrations of coffee waste (Fig. 5) indicated that increase in concentration to 18% increased the β-mannanase production by *P. citrinium* 150 Gy to 129 IU/ml, whereas the wild-type strain produced 16 IU/ml at the same concentration at optimum incubation periods using shaking technique.

Youssef *et al.* [25] revealed that the optimum coconut concentration was 5 g per 50 ml, yielding the highest mannanase activity (8.6 U/ml) and protein content.

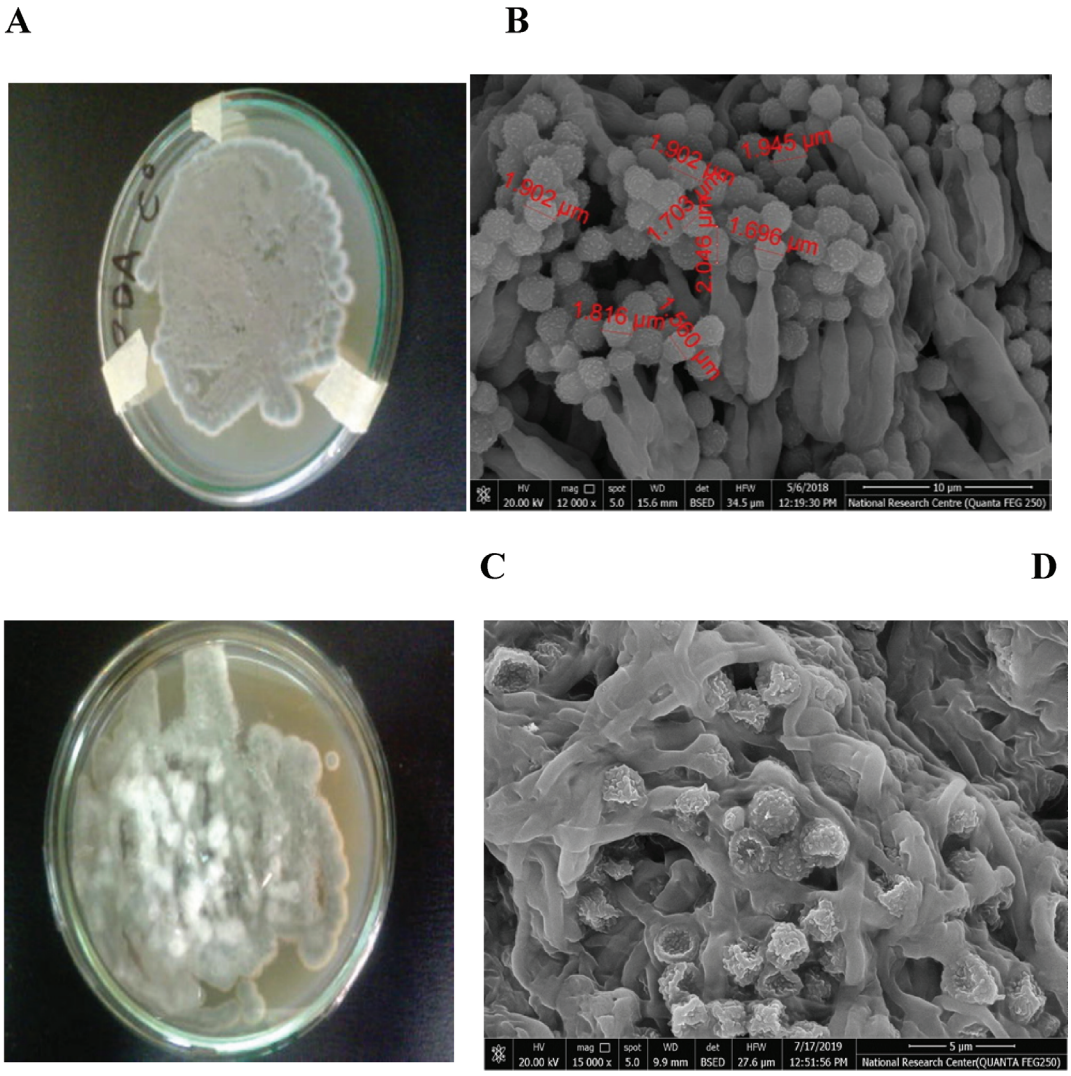
Effect of different nitrogen sources on mannanase production

The nitrogen source had a great influence of the biosynthetic pathways of the bioactive metabolites of the produced strain [26]. Different nitrogen sources were examined for the production of β-mannanase. On equivalent nitrogen base, ammonium sulfate, peptone, and urea of the basal medium were substituted individually by different nitrogen sources. These included organic sources, namely, baker's yeast, casein, corn steep liquor, peptone, urea, and yeast extract, and inorganic sources, namely, ammonium sulfate and sodium nitrate.

It was quite clear from the results in Fig. 6 that the mixed nitrogen source produced the highest level of β-mannanase for both *P. citrinium* and *P. citrinium* 150 Gy strains, which were 16.3 and 129.92 IU/ml, respectively.

El-Refai *et al.* [16] also recorded that the mixed nitrogen source was the best nitrogen source for production of *Penicillium humicola* β-mannanase.

Figure 3



Colonial and morphological characteristic of wild (a, b) and mutant (c, d) for most potent fungal isolate.

Saad *et al*. [27] reported that sodium nitrate was the best inorganic source for production of *Aspergillus tamarii* NRC3 β-mannanase.

Effect of different concentration of nitrogen

The production of β-mannanase in *P. citrinium* and *P. citrinium* 150 Gy mutant was increased up to 26.5 and 219 IU/ml as the concentration of nitrogen source of the culture medium increased to1.2 and 1.4%, respectively.

The β-mannanase production was almost stable by increasing the nitrogen concentration of the culture medium to 1.8% (Fig. 7).

Youssef *et al.* [25] indicate that the increase in β-mannanase production was observed with raising the NH_4Cl concentration to 0.25% and decreased gradually at higher concentrations.

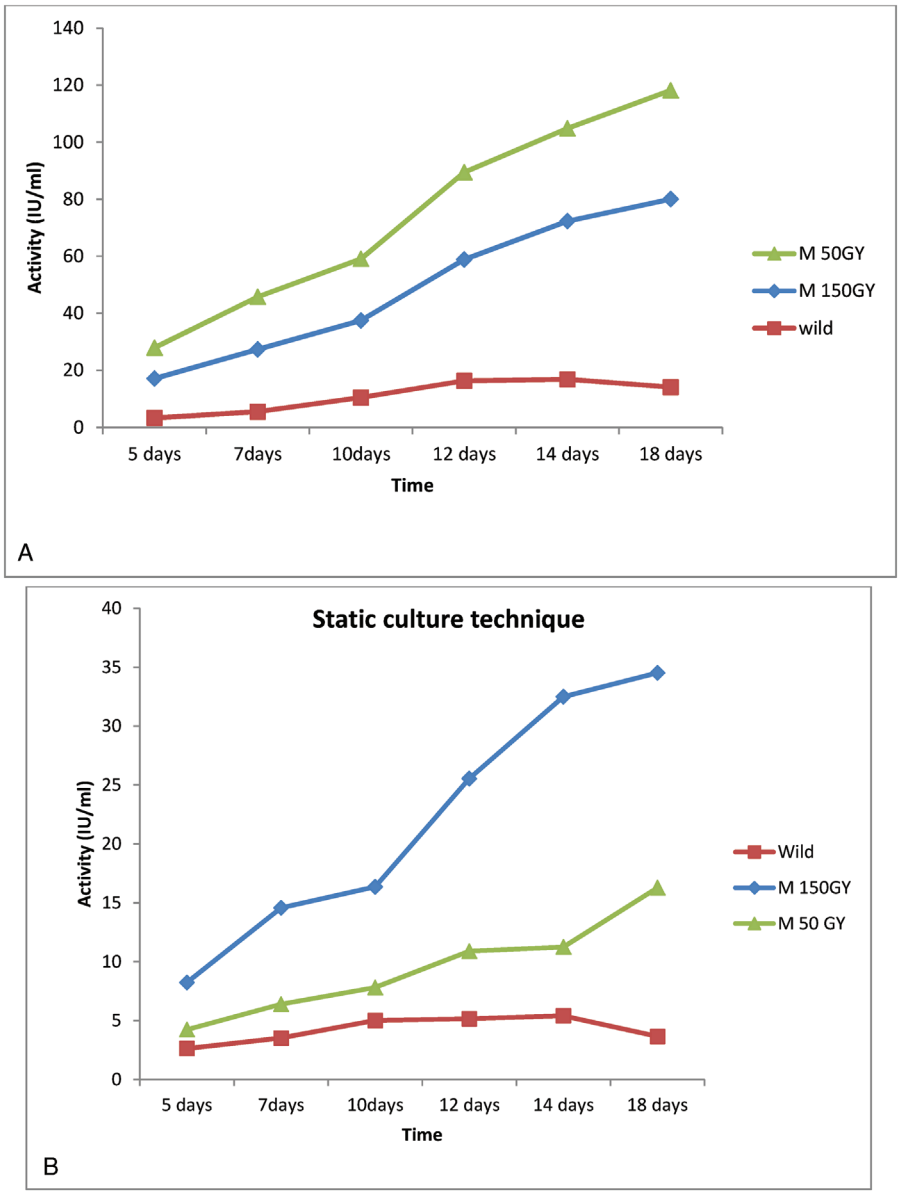
The aforementioned optimization producers increased the biosynthesis of β-mannanase by *P. citrinium* 150 Gy mutant strain to 219 IU/ml, which was higher by 8.26-fold from β-mannanase produced by the wild-type *P. citrinium* (26.5 IU/ml) after optimization. Therefore, *P. citrinium* 150 Gy mutant strain was used for statistical optimization.

Statistical optimization for the production of the mutant strain β-mannanase

(1) PBD

PBD was used to investigate the relative interaction and the variable of different parameters for the culture processing. Eleven trials for seven variables (Table 2) clarify the wide variation in the production of β-mannanase from 12.9 to 296 IU/ml, which implied the great influence of different factors in the fermentation

Figure 4



(a) Biosynthesis of extracellular β-mannanase using shaken culture medium at different incubation periods for wild and mutant strains; (b) biosynthesis of β-mannanase using in static culture medium at different incubation periods for wild and mutant strains.

process. The highest value of the β-mannanase 296 IU/ml was produced in trial 8, which contained the following: coffee waste, 10; nitrogen mixture, 1.8%; KH_2PO_4, 8.5; and inoculum, 5 ml, at 140 rpm, at pH 5 after 21 days. On the contrary, the lowest value of β-mannanase 296 IU/ml was produced in trial 6, which contained coffee waste, 10; nitrogen mixture, 1%; KH_2PO_4, 5.5; and inoculums, 5 ml at 100 rpm at pH 4 after 21 days.

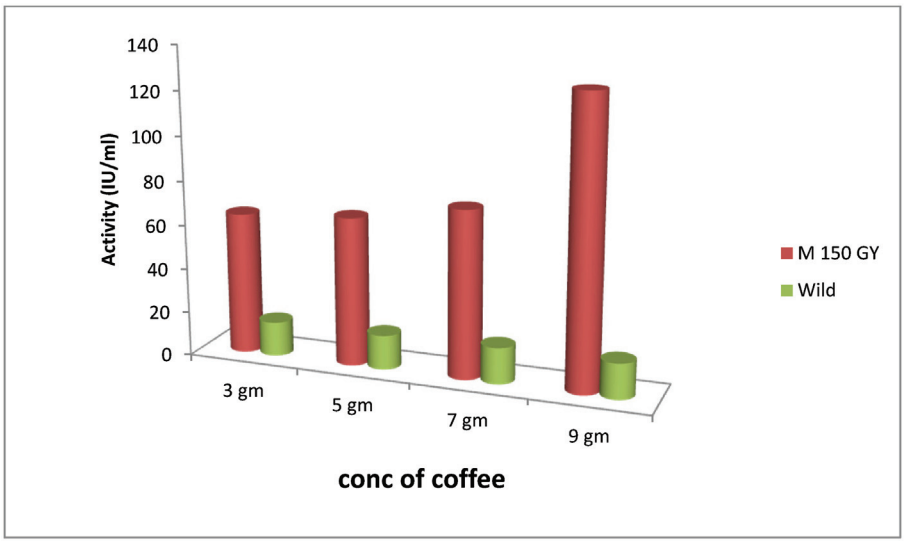
The main effects of the tested parameters on the production of β-mannanase were calculated and graphically represented in Fig. 8. All the examined factors except inoculum possessed positive effect.

Confidence level, *P*-effect and *t*-test of the statistical analysis of the PBD are indicated in Table 3. The variables showed high confidence level above 99% for nitrogen complex, KH_2PO_4, initial pH, and rpm, and they were selected for further optimization.

The first-order model describing the correlation of the seven factors and the β-mannanase activity could be presented as follows:

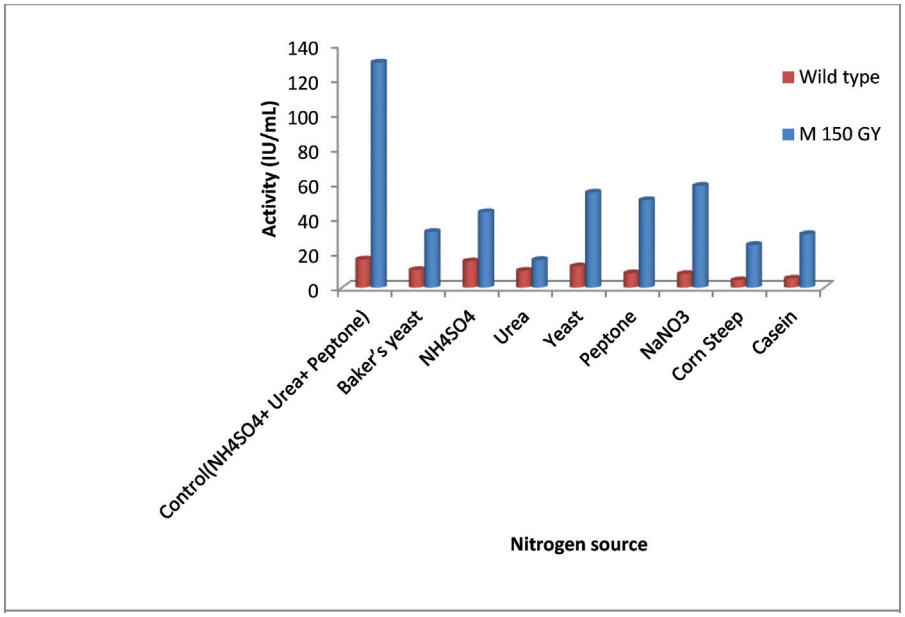
$$Y_{Activity} = -1514.040 + 28.667 \times_1 + 100.674 \times_2 + 47.125 \times_3 + 7.454 \times_4 + 5.454 \times_5 + 3.143 \times_6 + 77.473 \times_7$$

Figure 5



Effect of different concentration of carbon source on the production of wild and mutant extracellular β-mannanase.

Figure 6



Effect of different nitrogen source on the production of wild and mutant extracellular β-mannanase.

(2) RSM

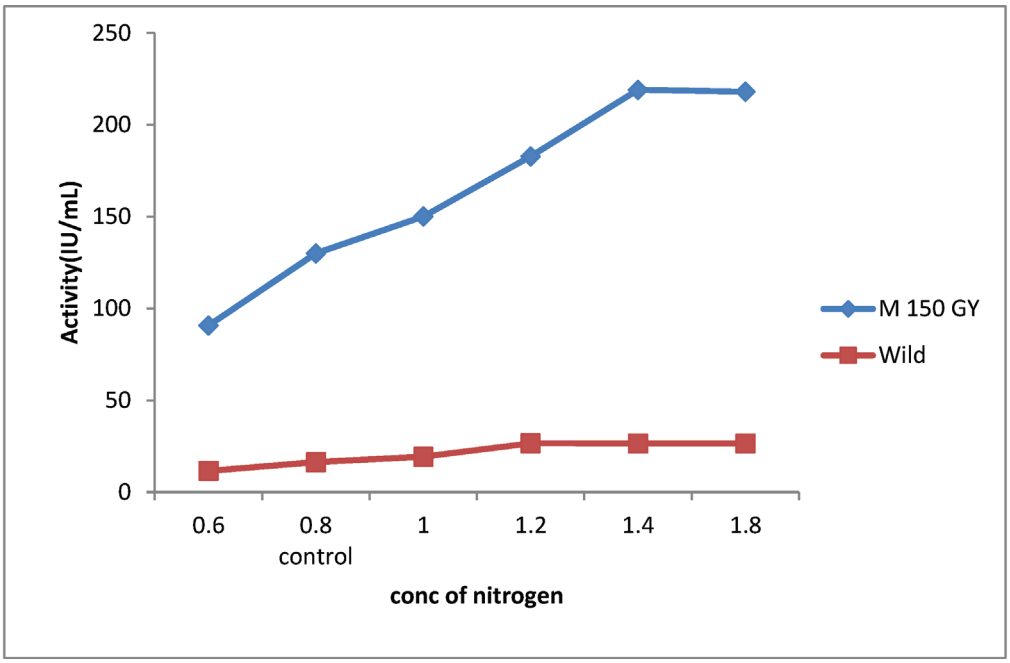
The optimal levels of the most effective variables (nitrogen complex, KH_2PO_4, initial pH, and rpm) arising from PBD were determined by applying RSM involving CCD in 30 trials (Table 4).

Culture medium containing in g/l, peptone (25), ammonium sulfate (19.15), urea (3.83), $MgSO_4 \cdot 0.7H_2O$ (0.6), K_2HPO_4 (8.5), and coffee waste(10 g/flask), adjusted at pH 5 (before autoclaving) inoculated by 8% of the inoculums and incubated at 30°C in a shaking incubator at 140 rpm for 21 days was used as the central point of the CCD.

The independent variables with their coded matrix and responses are listed in Table 4. In this table, experimental and predicted values for β-mannanase activity are presented. Variation in the enzyme yield from 65 to 351 IU/ml was observed during the 30 runs of the experiments. The highest level of the produced β-mannanase was 351 IU/ml obtained in run 5, which indicated that the optimal levels of the tested variable were as follows: KH_2PO_4, 8.5; rpm, 120; and nitrogen complex, 1.8% at pH5.0.

The determination coefficient (R^2) shows the accuracy of the model. The R^2 value is 0.9562, indicating that

Figure 7



Effect of different concentration of nitrogen source on the production of wild and mutant extracellular β-mannanase.

Table 2 Results for Plackett–Burman experiment

Trial	Carbon	Mixed nitrogen	KH_2PO_4	Time	Inoculum	RPM	PH	Enzyme activity (IU/ml)	Specific activity
1	8 (–1)	1 (–1)	6.5 (–1)	21 (+1)	5 (+1)	140 (+1)	4	26.35±3.59	2.24
2	10 (+1)	1 (–1)	6.5 (–1)	15 (–1)	3 (–1)	140 (+1)	5 (+1)	117±1.43	10.30
3	8 (–1)	1.8 (+1)	6.5 (–1)	15 (–1)	5 (+1)	100 (–1)	5 (+1)	19.27±7.00	1.81
4	10 (+1)	1.8 (+1)	6.5 (–1)	21 (+1)	3 (–1)	100 (–1)	4 (–1)	33.57±1.83	3.08
5	8 (–1)	1 (–1)	8.5 (+1)	21 (+1)	3 (–1)	100 (–1)	5 (+1)	69.58±5.22	8.03
6	10 (+1)	1 (–1)	8.5 (+1)	15 (–1)	5 (+1)	100 (–1)	4 (–1)	12.9±4.59	1.43
7	8 (–1)	1.8 (+1)	8.5 (+1)	15 (–1)	3 (–1)	140 (+1)	4 (–1)	136.1±7.27	10.84
8	10 (+1)	1.8 (+1)	8.5 (+1)	21 (+1)	5 (+1)	140 (+1)	5 (+1)	296±4	17.19
9	9 (0)	1.4 (0)	7.5 (0)	18 (0)	4 (0)	120 (0)	4.5 (0)	191±7.54	15.44
10	9 (0)	1.4 (0)	7.5 (0)	18 (0)	4 (0)	120 (0)	4.5 (0)	191±7.54	15.44
11	9 (0)	1.4 (0)	7.5 (0)	18 (0)	4 (0)	120 (0)	4.5 (0)	191±7.54	15.44

the 95.62% variability in the response is explained by the independent variables. Therefore, the present value of R^2 confirms the reliability of the current model for the production of β-mannanase and also exhibited a good correlation between the experiment and the theoretical values (Table 5).

A second-order polynomial equation used for the interpretation of correlation between variables and the response is presented as follows:

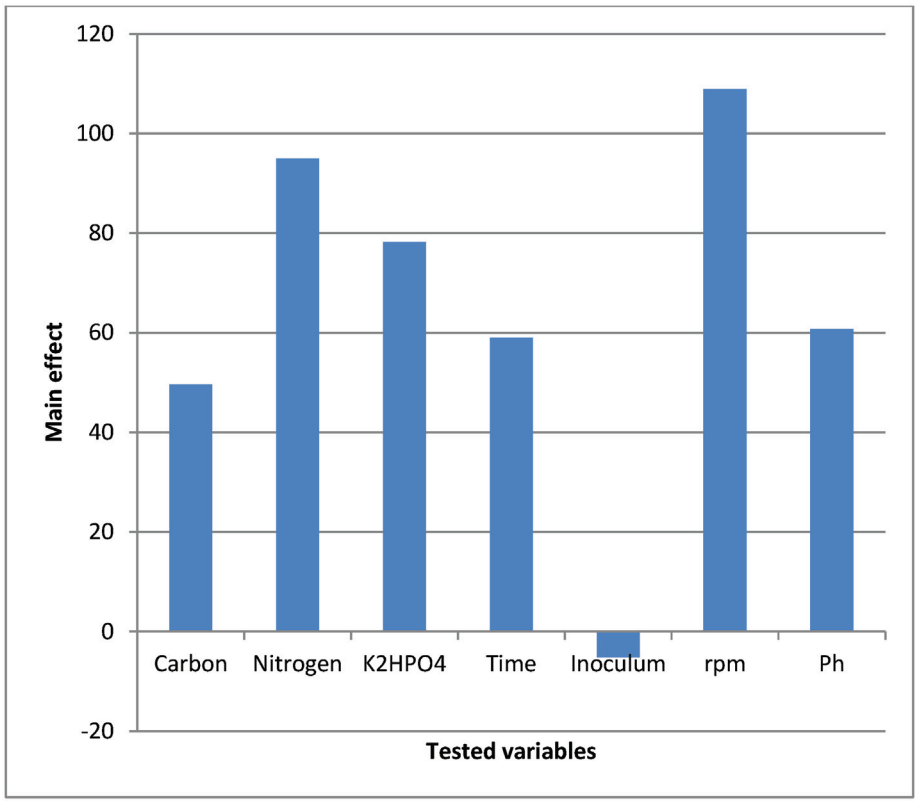
$$Y_{Activity} - 30832.4 + 4094.543 \times {}_1 + 72.56156 \times {}_2 + 3174.327 \times {}_3 + 2442.402 \times {}_4 - 2.09893 \times {}_1X_2 - 173.769 \times {}_1X_3 + 48.55852 \times {}_1X_4 + 8.656682 \times {}_2X_3 - 6.14703 \times {}_2X_4 - 8.91002 \times {}_3X_4 - 217.122 \times {}_1{}^2 - 0.16539 \times {}_2{}^2 - 784.118 \times {}_3{}^2 - 199.603 \times {}_4{}^2$$

Y, represents response or β-mannanase yield and X_1, X_2, X_3, and X_4 are KH_2PO_4, RPM, nitrogen complex, and pH, respectively.

The mathematical optimal point of the equation was 403.8 IU/ml at 120 rpm and 1.6975% of mixed nitrogen with 8.5 g/l of KH_2PO_4 at pH, 5. Three-dimensional graphs of the regression equation (Fig. 9) explained main and interaction effects of KH_2PO_4 and rpm; KH_2PO_4 and mixed nitrogen; KH_2PO_4 and pH; rpm and mixed nitrogen; rpm and pH; mixed nitrogen and pH on β-mannanase production by *P. citrinium* 150 GY mutant strain respectively.

The P value was used as a tool to check the significance of each coefficient, which in turn indicates the pattern of the interaction between the variables [28]. The

Figure 8



Main effects of independent variables on β-mannanase production by *Penicillium citrinium* according to the results of the Plackett–Burman design.

Table 3 Statistical analysis of Plackett–Burman design showing coefficient values, effect, *t* values, and *P* values for each variable on β-mannanase analysis

	Unstandardized coefficients		Standardized coefficients	*t*	*P* value	Confidence level (%)
	B	SE	*β*			
(Constant)	–1514.040	180.119		–8.406	0.000	
C	28.667	10.081	0.250	2.844	0.009 (HS)	99.9000
N	100.674	25.203	0.351	3.995	0.001 (HS)	99.99661
K	47.125	10.081	0.410	4.675	0.000 (HS)	99.99952
T	7.454	3.360	0.195	2.218	0.036 (S)	99.2222
Inc	5.454	10.081	0.047	0.541	0.593 (NS)	52.5000
RPM	3.143	0.504	0.547	6.236	0.000 (HS)	99.99999
pH	77.473	20.162	0.337	3.842	0.001 (HS)	99.99467

HS, highly significant; S, significant.

statistical analysis of data (Table 5) indicated high significant effect of terms with smaller *P* values ($P<0.05$) on β-mannanase production.

The results obtained by analysis of variance analysis (Tables 4 and 5) showed a significant *F* value (23.39547) which implied the model to be significant. Model terms having values of prob ? *F* (1.17*E*–07) less than 0.05 were considered significant.

It was reported by many researchers that the statistical optimization model for fermentation process could overcome the limitation of classic empirical methods and was proved to be more significance for the optimization production of β-mannanase [5,29–31].

Validation of the model

The validity of the proposed model was estimated by prediction of *P. citrinium* β-mannanase production for each trial of the matrix. The experimental results in Table 3 show that the maximum observed β-mannanase production (351 IU/ml) was very close to the predicted value (352 IU/ml).

Table 4 Central composite design and response β-mannanase activity by *Penicillium citrinium*

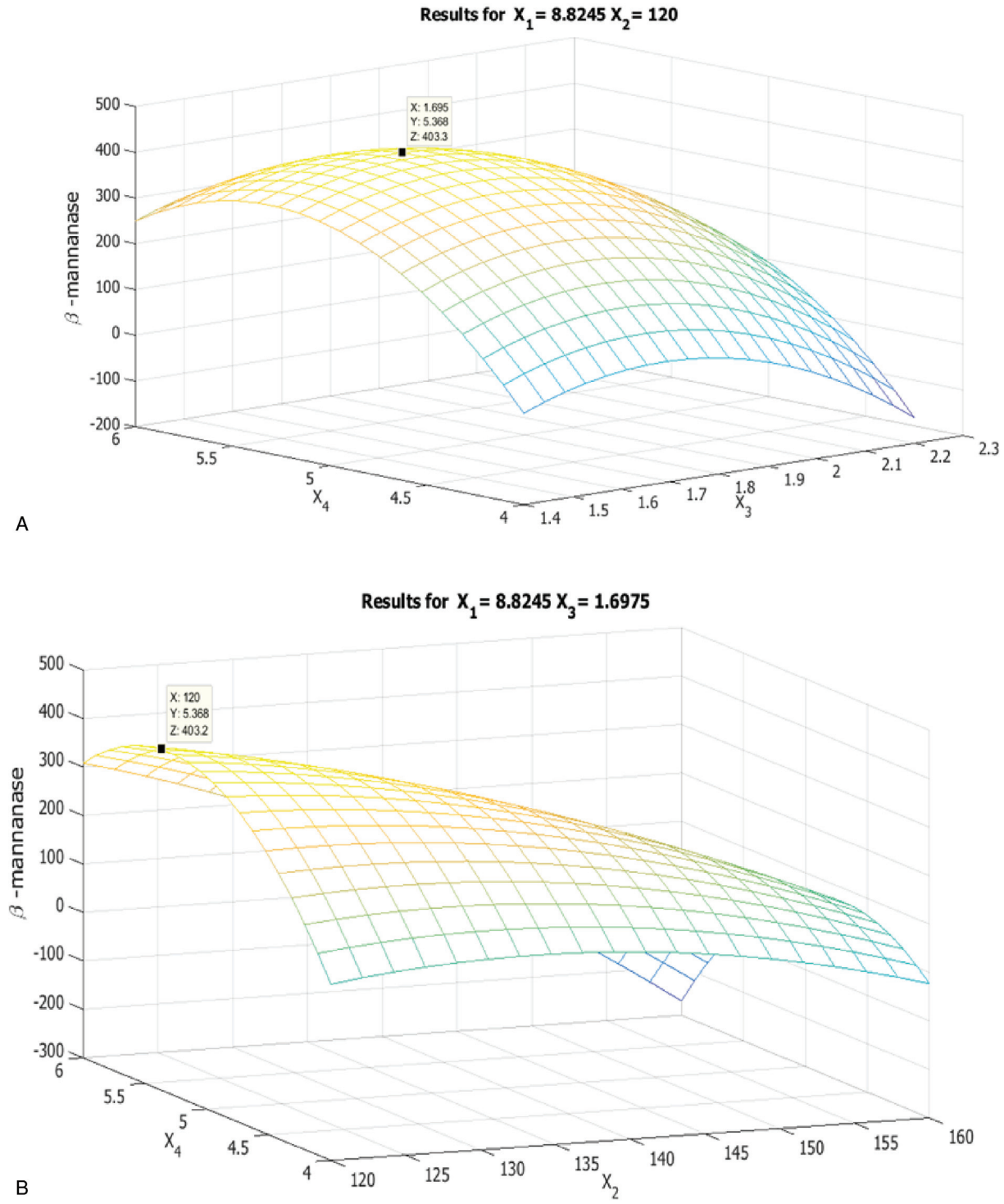
Trail	KH_2PO_4 (X_1)	RPM (X_2)	Mixed nitrogen (X_3)	pH (X_4)	β-mannanase activity	Predicted	Specific activity
1	9 (+1)	130 (−1)	2 (+1)	5.5 (+1)	258.82±2.03	254.5763	14.02818
2	8.5 (0)	140 (0)	1.8 (0)	6 (+2)	76.11±5.67	83.13624	4.676498
3	9 (+1)	130 (−1)	1.6 (−1)	5.5 (+1)	321±8.94	291.1784	17.32794
4	8.5 (0)	140 (0)	2.2 (+2)	5 (0)	248±4.35	212.9077	11.95181
5	8.5 (0)	120 (−2)	1.8 (0)	5 (0)	351±5.56	352.8814	21.56682
6	9.5 (+2)	140 (0)	1.8 (0)	5 (0)	100.3±8.03	124.5293	5.626928
7	8 (−1)	150 (+1)	1.6 (−1)	5.5 (+1)	11.34±1.09	−22.3742	0.549153
8	8 (−1)	130 (−1)	1.6 (−1)	5.5 (+1)	162.53±1.99	189.3489	8.136671
9	9 (+1)	150 (+1)	1.6 (−1)	5.5 (+1)	70.5±0.68	57.07876	3.477189
10	9 (+1)	150 (+1)	2 (+1)	4.5 (−1)	152±2.94	130.168	8.19407
11	8.5 (0)	140 (0)	1.8 (0)	4 (−2)	93±1.47	103.8858	5.369515
12	9 (+1)	130 (−1)	1.6 (−1)	4.5 (−1)	251.3±0.71	238.9697	12.53367
13	9 (+1)	150 (+1)	2 (+1)	5.5 (+1)	89.04±1.56	105.7682	4.939806
14	8.5 (0)	140 (0)	1.8 (0)	5 (0)	296±4	284.204	17.18931
15	8.5 (0)	140 (0)	1.8 (0)	5 (0)	296±4	302.0241	17.18931
16	8.5 (0)	160 (+2)	1.8 (0)	5 (0)	85±4.55	101.0347	4.557641
17	8.5 (0)	140 (0)	1.8 (0)	5 (0)	296±4	302.0241	17.18931
18	8 (−1)	150 (+1)	2 (+1)	5.5 (+1)	73.64±2.28	81.5668	3.996744
19	8 (−1)	130 (−1)	2 (+1)	4.5 (−1)	150.3±1.87	168.7081	8.246914
20	9 (+1)	130 (−1)	2 (+1)	4.5 (−1)	167.4±4.25	188.1116	8.868874
21	8.5 (0)	140 (0)	1.8 (0)	5 (0)	296±4	302.0241	17.18931
22	8.5 (0)	140 (0)	1.8 (0)	5 (0)	296±4	302.0241	17.18931
23	8 (−1)	150 (+1)	1.6 (−1)	4.5 (−1)	65±1.51	64.84014	3.851852
24	8 (−1)	130 (−1)	1.6 (−1)	4.5 (−1)	207±3.61	167.8787	11.14401
25	7.5 (−2)	140 (0)	1.8 (0)	5 (0)	67.63±3.02	61.3129	3.831728
26	9 (+1)	150 (+1)	1.6 (−1)	4.5 (−1)	115.11±3.90	93.95258	6.955287
27	8 (−1)	150 (+1)	2 (+1)	4.5 (−1)	168±1.51	184.8192	8.888889
28	8.5 (0)	140 (0)	1.4 (−2)	5 (0)	89±1.19	142.0047	5.274074
29	8 (−1)	130 (−1)	2 (+1)	5.5 (+1)	241.31±1.06	240.0745	13.20438
30	8.5 (0)	140 (0)	1.8 (0)	5 (0)	296±4	284.204	17.18931

Table 5 Analysis of Fractional Factorial design for β- mannanase activity by *Penicillium citrinium*

Regression statistics								
Multiple *R*	0.977859							
R^2	0.956209							
Adjusted R^2	0.915337							
SE	28.82681							
Observations	30							
ANOVA								
	d.f.	SS	MS	*F*	*Significance P*			
Regression	14	272 177.9	19 441.28	23.39547	1.17*E*−07			
Residual	15	12 464.77	830.9849					
Total	29	284 642.7						
	Coefficients	SE	*t*-Stat.	*P* value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	−30 832.4	3892.987	−7.91999	9.74*E*−07	−39 130.1	−22 534.7	−39 130.1	−22 534.7
X_1	4094.543	507.9082	8.06158	7.83*E*−07	3011.962	5177.123	3011.962	5177.123
X_2	72.56156	22.76043	3.188057	0.006111	24.04885	121.0743	24.04885	121.0743
X_3	3174.327	991.785	3.20062	0.005956	1060.387	5288.267	1060.387	5288.267
X_4	2442.402	386.8941	6.312844	1.39*E*−05	1617.757	3267.047	1617.757	3267.047
*X*1×2	−2.09893	1.474536	−1.42345	0.175073	−5.24182	1.043973	−5.24182	1.043973
*X*1×3	−173.769	72.06702	−2.41121	0.029178	−327.376	−20.1615	−327.376	−20.1615
X_1X_4	48.55852	29.49072	1.64657	0.120431	−14.2995	111.4165	−14.2995	111.4165
X_2X_3	8.656682	3.845649	2.251033	0.039809	0.459875	16.85349	0.459875	16.85349
X_2X_4	−6.14703	1.458992	−4.2132	0.000753	−9.25679	−3.03726	−9.25679	−3.03726
X_3X_4	−8.91002	5.656667	−1.57514	0.136077	−20.9669	3.146879	−20.9669	3.146879
X_1^2	−217.122	24.50007	−8.86209	2.39*E*−07	−269.343	−164.901	−269.343	−164.901
X_2^2	−0.16539	0.055042	−3.00488	0.008884	−0.28271	−0.04808	−0.28271	−0.04808
X_3^2	−784.118	142.9951	−5.48353	6.3*E*−05	−1088.91	−479.332	−1088.91	−479.332
X_4^2	−199.603	22.01684	−9.06592	1.79*E*−07	−246.531	−152.675	−246.531	−152.675

ANOVA, analysis of variance.

Figure 9



Explained main and interaction effects of (a) KH_2PO_4 and rpm, (b) KH_2PO_4 and mixed nitrogen, (c) KH_2PO_4 and PH, (d) rpm and mixed nitrogen, (e) rpm and PH, and (f) mixed nitrogen and pH on β-mannanase production by *Penicillium citrinium* 150 Gy mutant strain correspondingly.

Verification of the optimization models

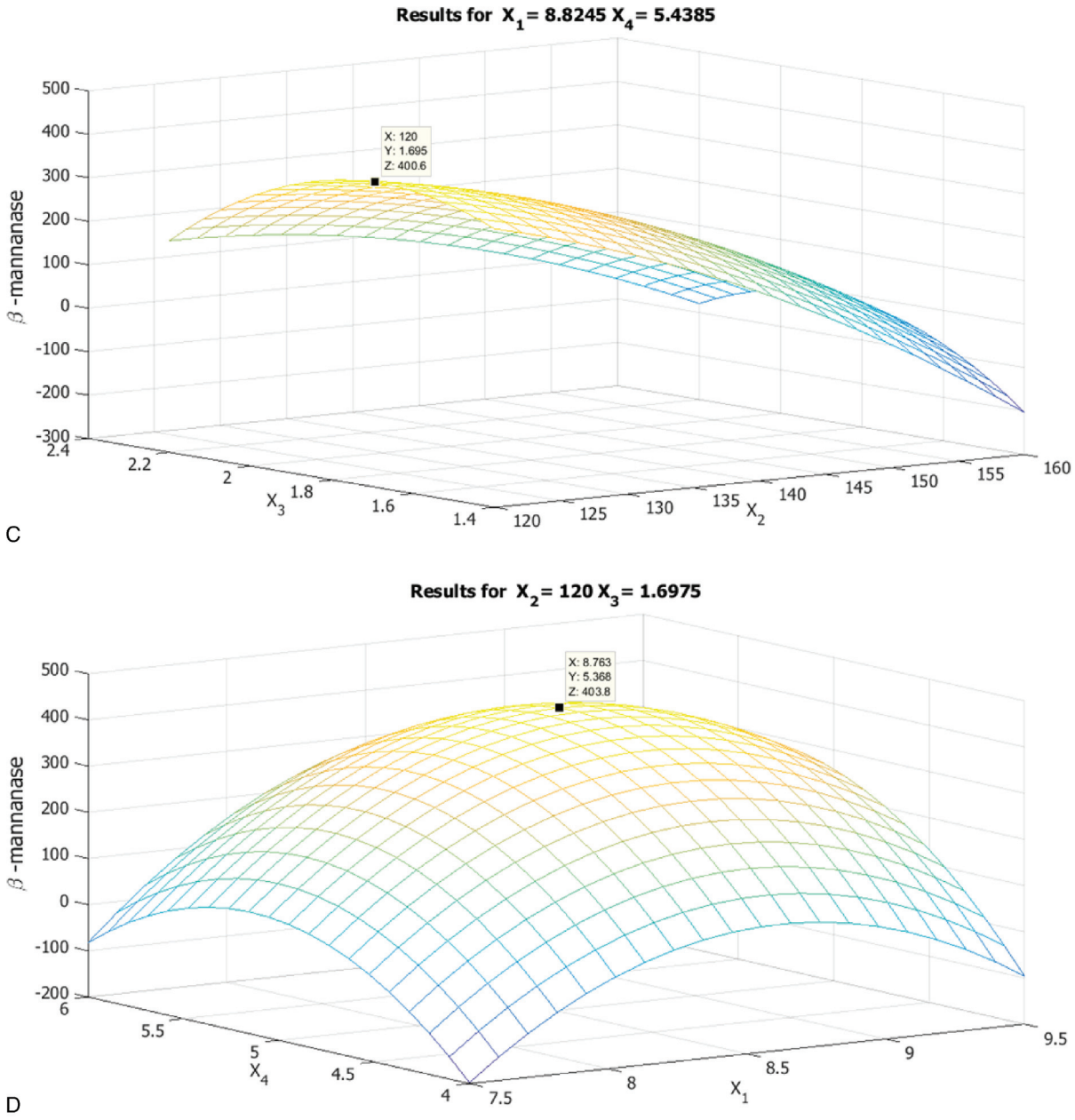
As shown in Table 6 under the optimization condition for *P. citrinium* 150-GY, β-mannanase was reached to 351 IU/ml with specific activity of 21.57. The results indicated that the optimized condition accelerated about 9.5-fold times than basal medium.

Primary characteristics of the crude wild and mutant *Penicillium citrinium* β-mannanase

Effect of pH of the reaction

The results in Fig. 10 show that *P. citrinium* and its *mutant* β-mannanase was optimally active at pH 5.5; below or above this pH, the activity decreased.

Figure 9



(*continued*)

In general, optimum pH for the activity of most fungal mannanases was in the acidic range [16,32].

Effect of temperature of the reaction mixture

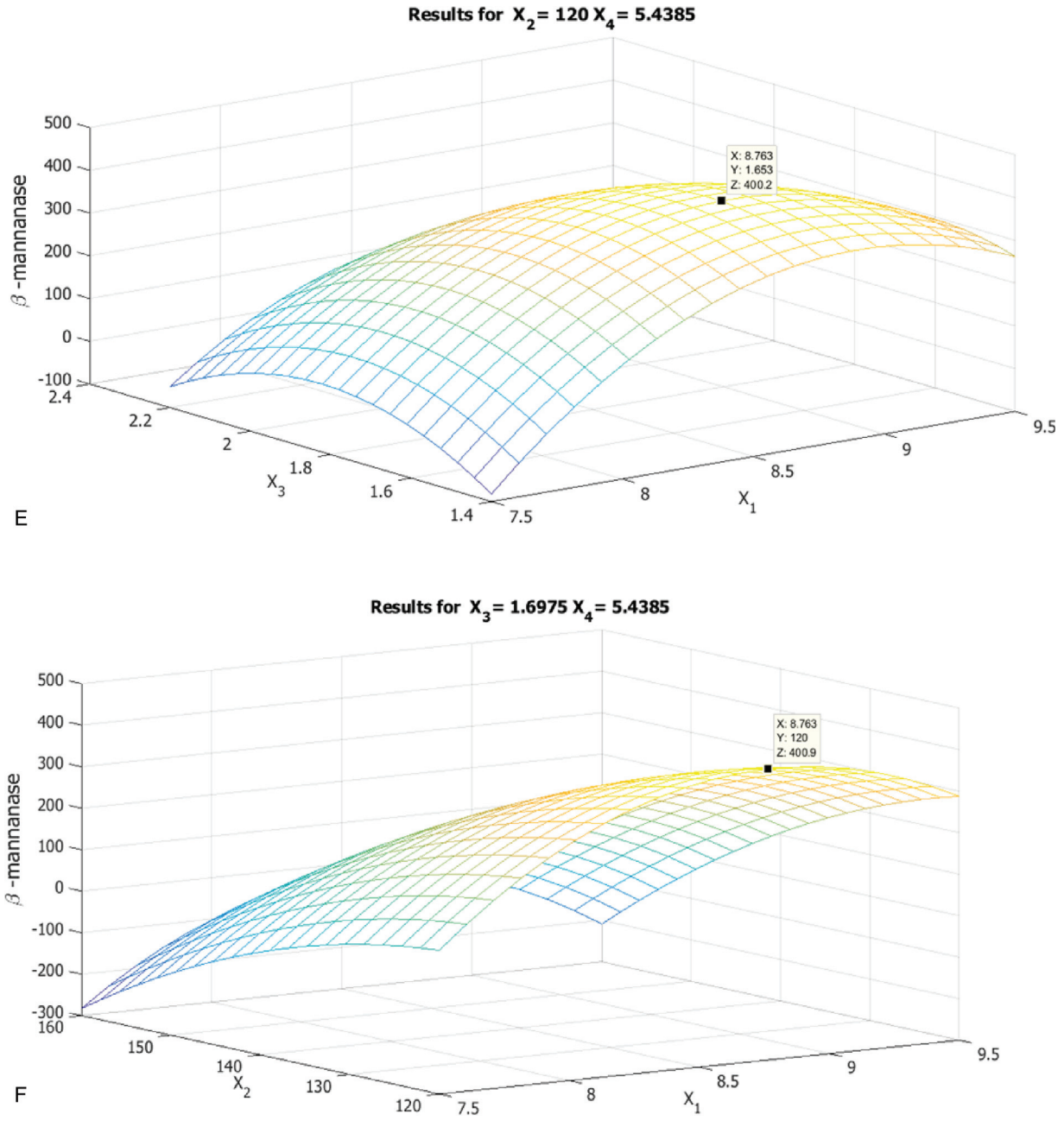
It was quite clear that β-mannanase produced from the mutant strain had optimum temperature at 60°C than that produced by the wild strain at 55°C; on the contrary, the gradual decrease in the temperatures around the optimum was slowly decreased for mutant one than the wild, as indicated in Fig. 11.

Chantorn *et al*. [33] showed that maximum activity of bacterial mannanase was at 50°C. There are several commercial advantages in carrying out enzymatic reactions at a higher temperature [34]. Enzymatic digestion at a high temperature (60–65°C) may reduce microbial contamination of the material being processed. In addition, higher temperatures increase the rate of substrate digestion and increase the solubility of the polymeric substrates such as carbohydrates, rendering them more amenable to enzymatic attack [35].

Thermal stability

The crude enzyme solution of wild strain and its mutant strain was incubated in the absence of its

Figure 9



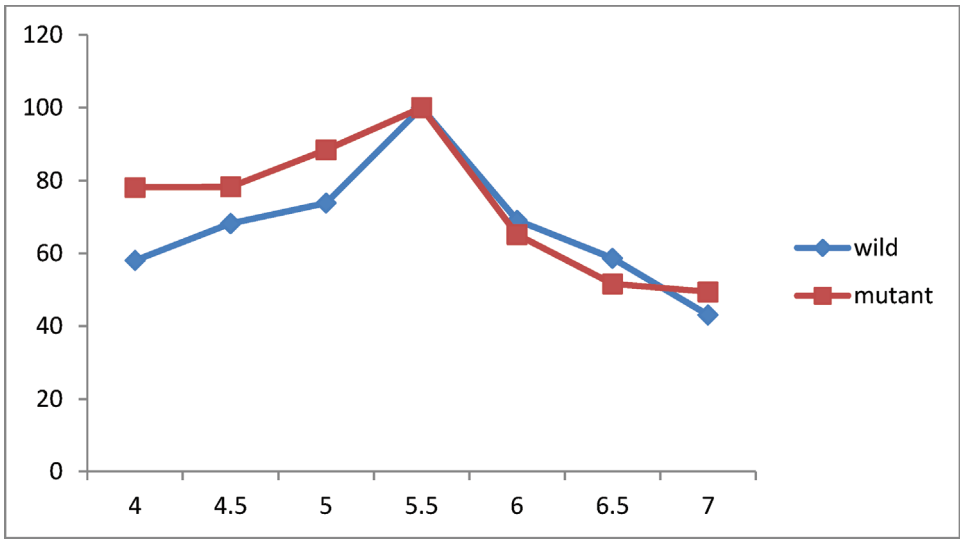
(*continued*)

Table 6 A verification experiment demonstrating the optimization steps targeted β-mannanase biosynthesis by wild and mutant *Penicillium citrinium*

Response	*Penicillium citrinium*	Condition			
		Penicillium citrinium 150 Gy strains	One variable at a time	Plackett–Berman design	Central composite design
β-mannanase activity	16.3	37	191	296	351
Specific activity	2.29	4.99	16.28	17.19	21.57

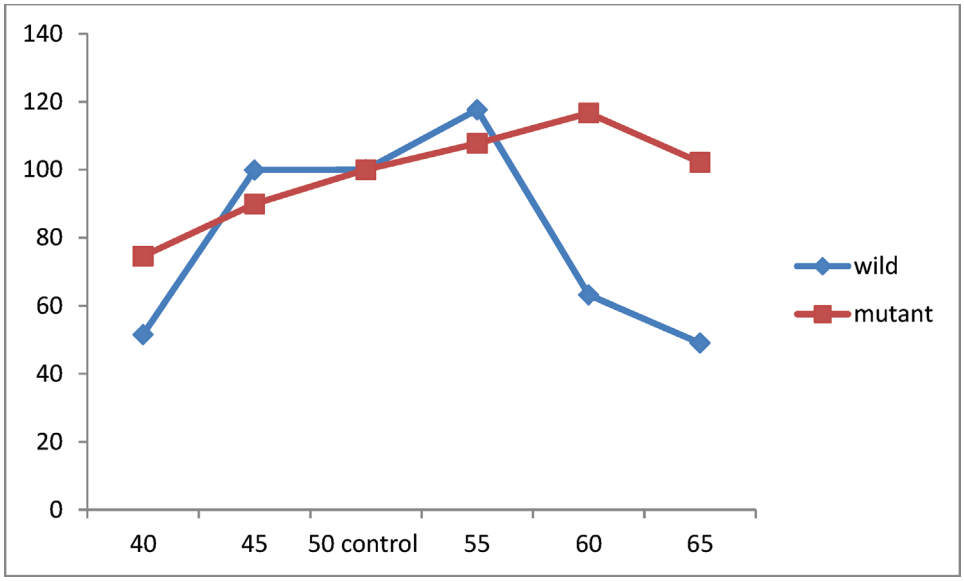
substrate at 40, 45, 50, and 55°C in a water bath, and the residual activities at different periods up to 2 h were determined under the optimum conditions (pH 5.5, 55°C) for wild and (pH 5.5, 60°C) for mutant. After 2 hr, the crude enzyme in the wild type retained 97.1% of its activity at 40°C and 94.35% at 45°C, but in the mutant, the crude enzyme kept its activity, and there is no any loss at 40°C and retained 95.4% after 2 h (Fig. 12).

Figure 10



Effect of pH of the reaction mixture on the crude *Penicillium citrinium* β-mannanase. Control: at pH 5.0 represent 100%.

Figure 11



Effect of temperature of the reaction on the crude *Penicillium citrinium* β-mannanase. Control: at pH 5.5 and temperature 50°C represent100%. (a)Thermal stability of crude enzyme (wild); (b)Thermal stability of crude enzyme (mutant)

After heating the crude enzyme of wild and mutant at 50°C up to 15 min, most of the activity was retained (96.86 and 98.45%, respectively), and still retained 71.35 and 86.21%, respectively, of its activity after 60 min At 50°C, the *P. citrinium* and *P. citrinium* 150 GY mutant strain retained 71.35 and 86.21%, respectively, of its activity after 60 min

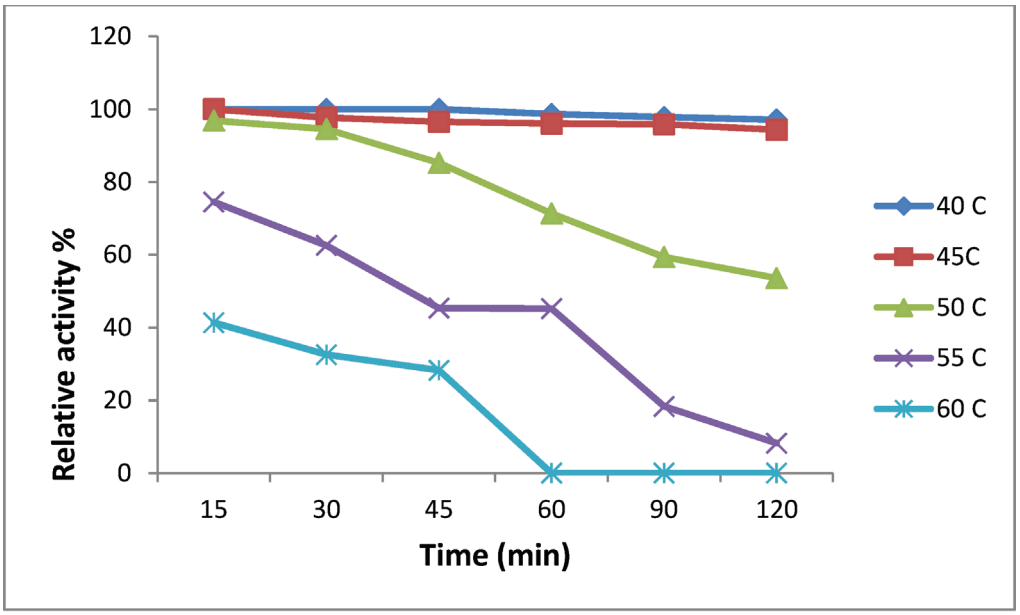
At 55°C, the thermostability of both enzymes was almost equal after 1 h (74%).

The produced β-mannanase from both wild and mutant strains was quite stable than that recorded by El-Refai *et al.* [16], which recorded that *Penicillium humicola* β-mannanase lost ~20% of its activity after one hour at 50°C.

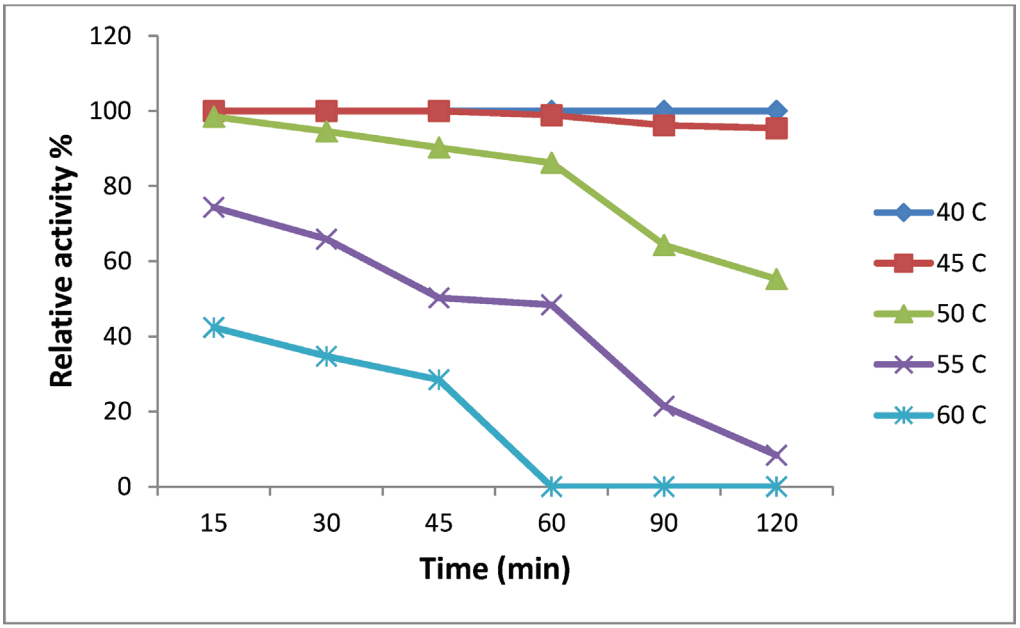
Conclusion

In our work, we found that gamma mutant was stable at 150 Gy. The first step of the fermentation optimized by one-factor-at-a-time technique increased the biosynthesis of β-mannanase for *P. citrinium* 150 Gy from 65.9 to 219 IU/ml comparing with the wild strain, which increased from 16.82 to 26.5 IU/ml.

Figure 12



Thermal stability of crude enzyme (wild)



Thermal stability of crude enzyme (mutant)

(a, b) Thermal stability of the crude *Penicillium citrinium* β-mannanase.

Statistical optimization improved *P. citrinium* 150 Gy β-mannanase level from 219 to 296 IU/ml by applying PBD and increased the level of β-mannanase biosynthesis to 351 IU/ml. Primary characterization of β-mannanase produced by *P. citrinium* and *P. citrinium* 150 Gy proved that they are almost the same except in a little shift to higher value (5°C) in optimum temperature.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

References

1 Srivastava PK, Kapoor M. Production, properties, and applications of endo-β-mannanases. Biotechnol Adv 2017; 35:1–19?

2 Abdel-Fattah AAF, Hashem AM, Ismail AMS, Refai EMA. Purification and some properties of β-mannanase from *Aspergillusoryzae*NRRL 3448. J App Sci Res 2009; 5:2067–2073.

3 Mannanase KS. Microbial Enzymes in Bioconversions of Biomass. Bangkok: Department of Biotechnology, Faculty of Agro-Industry, Kasestsart University; 2016; 215–229.

4 Germec M, Yatmaz E, Karahalil E, Turhan I. Effect of different fermentation strategies on β-mannanase production in fed-batch bioreactor system. Biotech 2017; 7:77.

5 Ismail SA, Khattab OKH, Nour SA, Awad GE, Abo-Elnasr AA, Hashem AM. A thermodynamic study of partially-purified *Penicilliumhumicola* β-mannanase produced by statistical optimization. Jordan J Biol Sci 2019; 12.

6 Mandal A. Review on microbial xylanases and their applications. Int J Life Sci 2015; 4:178–187.

7 Burlacu A, Israel-roming F, Cornea CP. Fugal strains improvement for xylanase over production through physical and chemical mutagenesis. Agrolife Sci J 2017; 16:40–47.

8 Uday USP, Choudhury P, Bandopadhyay TK, Bhunia B, B. Classification, mode of action and production strategy of xylanase and its application for biofuel production from water hyacinth. Int J Biol Macromolecules 2016; 82:1041–1054.

9 Ghazi S, Sepahy AA, Azin M, Khaje K, Khavarinejad R. UV mutagenesis for the overproduction of xylanase from *Bacillus mojavensis* PTCC 1723and optimization of the production condition. Iran J Basic Med Sci 2014; 17:844–853.

10 Hanim C, Yusiati LM, Cahyanto MN, Wibowo A. Mutagenic improvement of xylanaseproduction from xylanolytic bacteria and its phylogenetic analysis. Microbiol Indonesia 2013; 7:51–58.

11 Khodayari F, Cebeci Z, Ozcan BD. Optimization of xylanase and α-amylase production by alkaline and thermophilic *Bacillusisolate* KH-13. J Entomol Zool Stud 2014; 2:295–303.

12 Baraldi IJ, Giordano RLC, Zangirolami TC. Enzymatic hydrolysis as an environmentally friendly process compared to thermal hydrolysis for instant coffee production. Brazil J Chem Engineering 2016; 33:763–771.

13 El-Bondkly AM, Keera AA. UV-and EMS-induced mutations affecting synthesis of alkaloids and lipase in *Penicilliumroquefortii*. Arab J Biotechnol 2007; 10:241–248.

14 Shahbazi S, Ispareh K, Karimi M, Askari H, Ebrahimi MA. Gamma and UV radiation induced mutagenesis in *Trichodermareesei*to enhance cellulases enzyme activity. Int J Fam Alli Sci 2014; 3:543–554.

15 Plengvidhya V, Breidt FJr, Fleming HP. Use ofRAPD-PCR as a method to follow the progress of starter cultures in sauerkraut fermentation. Int J Food Microbiol 2004; 93:287–296.

16 El-Refai MA, Khattabk OH, Ismail SA, Hashem AM, Abo-Elnasr AA, Nour SA. Improved mannanase production from *Penicilliumhumicola*and application for hydrolysis property. Egyptian Pharm J 2014; 13:160–167.

17 Hashem AM, Ismail AMS, El-Refai MA, Abdel-Fattah AF. Production and properties of β-Mannanase by free and immobilized cells of *Aspergillusoryzae*NRRL 3488. Cytobios 2001; 105:115–130.

18 Smogi M. Notes on sugar determination. J Biol Chem 1952; 195:19–23.

19 Lowry OH, Rosebrough NH, Farr AL, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265–275.

20 Plackett RL, Burman JP. The design of optimum multi-factorial experiments. Biometrika 1946; 33:305–325.

21 Box GE, Hunter WG, Hunter JS. Statisticsfor Experiments. New York, NY: Wile; 1978; 291–334

22 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 2013; 30:2725–2729.

23 Iftikhar T, Niaz M, Abbas SQ, Zia MA, Ashraf I, Lee KJ, Haq I. Mutation induced enhanced biosynthesis of lipases by *Rhizopusoligosporus*var. Microspores 2010; 42:1235–1249.

24 Olaniyi OO, Igbe FO, Ekundayo TC. Optimization studies on mannanase production by Trichosporonoidesoedocephalis in submerged state fermentation. J Biotechnol Pharm Res 2013; 4:110–116.

25 Youssef SA, El-Naggar MY, El-Assar SA, Beltagy EA. Optimization of cultural conditions for β-mannanase production by a local *Aspergillusniger* isolate. Int J Agri Biol 2006; 8:539–545.

26 Gesheva V, Ivanova V, Gesheva R. Effect of nutrients on theproduction of AK-111-81 macrolide antbiotic by *Streptomycehygroscopicus*. Microbiol Res 2005; 160:243–248.

27 Saad AM, Saad MM, Hassan HM, Ibrahim NA, El-Hadedy DE, Ibrahim EI, El-Din AL, Zahraa A. Optimization study for beta-mannanase production from locust bean gum by a local *Aspergillustamarii NRC3* isolate. Res J Pharm Biol Chem Sci 2016; 7:2597–2609.

28 Bahçeci KS, Acar J. Modeling the combined effects of pH, temperature and ascorbic acid concentration on the heat resistance of *Alicyclobacillu sacidoterrestis*. Int J Food Microbiol 2007; 120:266–273.

29 Ahirwar S, Soni H, Rawat HK, Prajapati BP, Kango N. Experimental design of response surface methodology used for utilization of palm kernel cake as solid substrate for optimized production of fungal mannanase. Mycol 2016; 7:143–153.

30 Janveja C, Rana SSA, Soni SK. Statistical optimization of media components for the enhanced β-xylanase and β-mannanase production from *Aspergillusniger*C-5 via solid state fermentation of wheat bran. Int J Pharm Bio Sci 2016; 7:89–102.

31 Soni H, Rawat HK, Ahirwar S, Kango N. Screening, statistical optimized production, and application of β-mannanase from some newly isolated fungi. Engin Life Sci 2017; 17:392–401.

32 Adesina CF, Oluboyede AO, Agunbiade SO, Aderibigbe OB, Kolade OH, Oluwole ME. Production and characterization of fungal extracellular β-mannanase. Am J Res Commun 2013; 2325–4076

33 Chantorn S, Natrchalayuth S, Phannachet K, Apiraksakorn J. Identification of suitable condition for mannanase production by *Bacillus spp*. GA2 (1). British Biotechnol J 2015; 5:92.

34 Ward OP, Moo-Young M. Enzymatic degradation of cell wall and related plant polysaccharides. Crit Rev Biotechnol 1989; 8:237–274.

35 Brock TD. Introduction, an overview. Thermophiles: general, molecular and applied microbiology. New York, NY: John Wiley & Sons Inc; 1986; 1–16