Optimization and molecular characterization of novel *Aspergillus* spp. producing invertase enzyme

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Background and objective
Invertase, one of the most important enzymes used in industrial and pharmaceutical applications, has been produced from different *Aspergillus* spp. Statistical and mathematical tools [response surface methodology (RSM)] have been carried out to optimize culture conditions to facilitate maximum enzyme production. This investigation aims to screen the ability of some *Aspergillus* spp. to produce invertase enzyme, to optimize the culture parameters, and to detect the genetic diversity between them.
Materials and methods
A total of five fungal strains were tested for the production of invertase. The most promising strain was selected for optimization. Optimization experiments were carried out to obtain maximum enzyme production, and a molecular marker (RAPD-PCR) was used to differentiate between these strains.
Results and conclusion
Some *Aspergillus* spp. were examined to produced invertase enzyme after 2 days of cultivation. *Aspergillus niger* recorded the highest enzyme production (6.8 U/ml). Ten RAPD-PCR primers were used to differentiate between *Aspergillus* spp. A total of 131 bands were amplified in the five strains of *Aspergillus* spp. Based on RAPD-PCR analysis, the highest similarities obtained between *A. parasiticus* and *A. niger* showed 75%. Moreover, the RAPD-PCR technique is a good tool for differentiation between the *Aspergillus* spp. To optimize culture condition, RSM was used to increase invertase production by *A. niger.* Temperature, pH, and incubation time dramatically affect invertase yield. A combination of these factors (pH 5, 35°C, and incubation for 72 h) was optimum for maximum production of invertase (8.2 U/ml). Our results employed RSM to elucidate the combination of effective factors such as temperature, pH, and incubation time to obtain a high yield of an important enzyme, invertase, from *A. niger*, used as a promising candidate for industrial applications.

Keywords:
Aspergillus spp, invertase, optimization and molecular characterization, RAPD-PCR, response surface methodology

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Introduction

β-Fructofuranosidase (EC.3.2.1.26) is the official name for invertase, which implies that the hydrolysis of the terminal non-reducing β-fructofuranoside residues in the β-fructofuranosides reaction is catalyzed by this enzyme [1]. Invertase is considered the most important enzyme that is produced from different organisms, such as yeast, bacteria, and filamentous fungi as good producers for invertase enzyme, which is used in various industrial applications with potential effects. It is used in fondants, soft-centered sweets, lactic acid, alcoholic drinks, and artificial honey industries owing to its sweetening effects. The production of inverted sugar is one of invertase's numerous applications owing to its sweetening effects, which are more than sucrose [2]. Other applications of this enzyme are seen in drug and pharmaceutical industries; moreover, the enzyme acts as an antioxidant, an immune booster, and used in treating some cases of cancer such as stomach and bone [3,4].

The optimization of the enzyme production in general and invertase production in particular includes fermentation conditions to obtain high enzyme yield and high microbial growth. Classical optimization methods involve identifying the various factors that may influence enzyme yield, and then, varying one of the factors at the same time keeping the rest constant. This methodology is a single-dimensional inquiry procedure, which though simple, does not achieve optimized conditions as it fails to consider interactions between factors. Therefore, in finding solutions to these obvious challenges, scientists designed statistical and mathematical procedures that circumvent these shortcomings. One of such statistical and mathematical procedure is response surface

methodology (RSM). RSM is an experimental approach that efficiently runs ideal conditions for multivariable strategy [5]. According to Ting *et al.* [6], RSM is an assemblage of mathematical and analytical procedures that design experiments, build models, measure responses, evaluate effects and relationship between groups of controlled factors under experimental conditions, and search for the optimum conditions in bioprocess optimization. In this study, RSM has been applied to optimize cultural conditions for the overproduction of invertase by *Aspergillus niger* grown on PDP. Molecular markers provide additional tools for characterization, differentiation, assessment of genetic relatedness, and diversity in collections. Among all molecular markers, RAPD-PCR technique of DNA fingerprinting has been used in this work, which is widely used in conservation biology because of its quick results, cost-effectiveness, easy analyzed results, and reproducibility [7].

Materials and methods

Fungal strains and culture conditions

Five isolates of *Aspergillus* spp. were used in this study: *A. niger*, *A. terreus*, *A. flavus*, *A. ochraceus*, and *A. parasiticus*. These strains were cultivated in potato dextrose agar (Merck KGaA, Germany) at 28°C for 4 days and kept at 4°C. Three isolates were previously identified in microbial genetic department, NRC, by Salim *et al.* [8] as *A. ochraceus Egy2* LC360803, *A. flavus* Egy3 under accession number LC368455, and *A. niger* Egy4 LC368456, and two isolates were friendly provided from the toxin department NRC. The experiments were performed using a production medium according to Dinarvand *et al.* [9]. It was slightly modified by adding sucrose 30 g/l, yeast extract 2.0 g/l, $NaNO_3$ 2 g/l, $MgSO_4$ 0.05 g/l, and K_2HPO_4 0.5 g/l, before sterilization at 121°C for 15 min. Erlenmeyer flasks (250 ml) containing 50 ml of the production medium were inoculated with stock culture and incubated at 30°C with 150 rpm shaking for 48 h.

Invertase assay

Invertase activity was assayed according to Miller [10] after 10 min of incubation of 0.2 ml enzyme solution with 1.8 ml of 1% sucrose in 0.2 M sodium acetate buffer (pH 5) at 50°C. Reducing sugars were quantified by addition of 1 ml of dinitrosalicylic acid to stop the reaction; 1 ml of dinitrosalicylic acid was added in boiling water for 10 min. Finally, absorbance was read at 540 nm in spectrophotometer (JASCOV-730; Jasco, Tokyo, Japan). One unit of invertase was defined as the amount of enzyme that catalyzed 1 μmol of reducing sugar/min.

Experimental design for response surface methodology and statistical analysis

In this study, three autonomous factors (temperature, pH, and incubation time) were represented at two levels, high concentration (+1) and low concentration −1), in twenty runs, as shown in Table 2. Each row represents a trial run, and each column represents an independent variable concentration. With the insignificant ones eliminated, a smaller and more fitting collection of factors was obtained and analyzed.

The design of 20 experiments and the coded and un-coded levels of the three investigated independent variables are listed in Table 2. The second-order observed model can be achieved from the experimental data, and the relationship between the response yield (invertase activity) and the variables through polynomial regression analysis. The form of the second-order polynomial model is as follows:

$$Y_{Activity} = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3 + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{23}X_2X_3 + ß_{11}X_1^2 + ß_{22}X_2^2 + ß_{33} + X_3^2$$

where $YActivit_y$ is the predicted production of invertase (U/ml) and *X1, X2,* and *X3* are the independent variables corresponding to the chosen affecting factors. $ß_0$ is the intercept; $ß_1$, $ß_2$, and $ß_3$ are linear coefficients; $ß_{11}$, $ß_{22}$ and $ß_{33}$ are quadratic coefficients; and $ß_{12}$, $ß_{13}$ and $ß_{23}$are cross-product coefficients [11].

Statistical analysis

The statistical analysis of the model was performed to assess the analysis of variance. Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the assessment of multiple coefficients for each variable. The quadratic models were showed as contour plots (3D), and the response surface curves were generated by using STATISTICA (0.6) (STATSOFT).

Molecular characterization of fungal strains

Extraction of genomic DNA

Using the DNeasy plant mini kit (Qiagen Sciences, Maryland, USA), extraction of genomic DNA was performed by following the manufacturer's instruction manual cat No.69104.

Differentiation of Aspergillus spp. using RAPD-PCR analysis

A total of 10 RAPD primers were used in the detection of polymorphism among the *Aspergillus* spp. These primers were synthesized by Metabion Corp. (Planegg-steinkirchen, Germany). The nucleotide

sequences and primers code are presented in Table 1. The amplification reactions of PCR were carried out as mentioned by Adawy *et al.* [12]. Reactions were performed in 25-µl volume composed of 1x reaction buffer, 0.2 mmol/l of dNTPs, 1.5 mmol/l $MgCl_2$, 0.2 µmol/l of primer, 0.5 unit of *Taq* polymerase (Qiagen Ltd, Germany), and 50 ng of template DNA, in sterile distilled water. PCR amplification of the DNA was performed in a Perkin Elmer thermal cycler 2400 programmed to fulfill 40 cycles. The temperature profile in the different cycles was as follows: an initial strand separation cycle at 94°C for 5 min followed by 40 cycles comprised of a denaturation step at 94°C for 1min, an annealing step at 36°C for 1 min, and an extension step at 72°C for 2 min. The final cycle was a polymerization cycle for 7 min at 72°C. PCR products were mixed with 2-µl gel loading dye and resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 mg/ml) in 1× TBE buffer at 120 volts. A 100-bp DNA ladder (promega) was used as a molecular size standard. PCR products were visualized under UV light and documented using a XR+ Gel Documentation System (Bio-Rad, Hercules, California, USA).

Table 1 The RAPD-PCR primer name and sequence

Primer	Sequence (5'–3')
A-05	5'-AGGGGTCTTG-3'
A-06	5'-GGTCCCTGAC-3'
A-08	5'-GTGACGTAGG-3'
A-20	5'-GTTGCGATCC-3'
B-07	5'-GGTGACGCAG-3'
B-09	5'-TGGGGGACTC-3'
B-12	5'-CCTTGACGCA-3'
B-17	5'-GGAGGGTGTT-3'
C-03	5'-GGGGGTCTTT-3'
C-05	5'-GATGACCGCC-3'

Data analysis

The amplified fragments were scored as present (1) or absent (0). Ladder 100-bp DNA was used to identify the molecular weights of fragments. Among the fungal isolates, the similarity matrix was calculated according to Coelho [13] and Rohlf [14], Unweighted Pair-Group Method with Arithmetical average was used to design the dendrogram for similarity coefficient.

Results

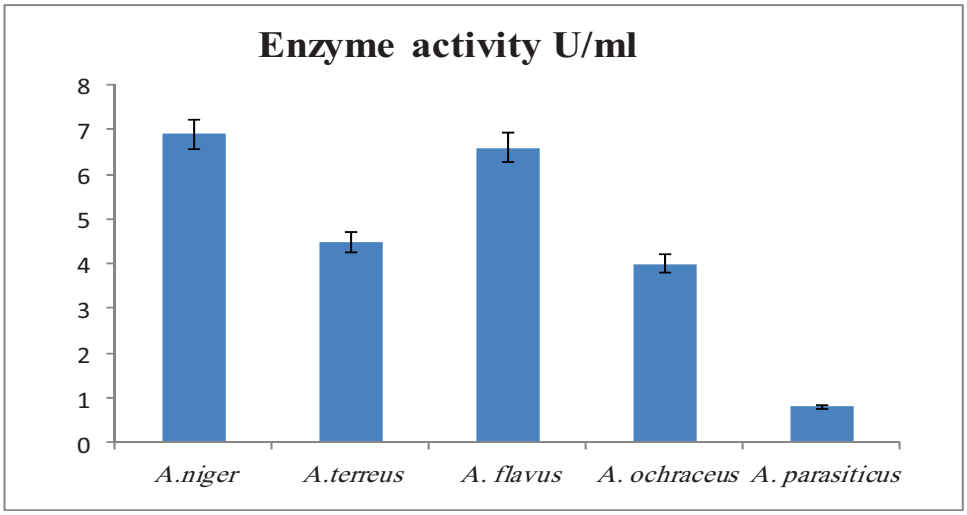
Screening of some *Aspergillus* spp. for invertase enzyme production

Among five *Aspergillus* spp., *A. niger* presented the highest invertase production (6.9 U/ml) after 48 h of cultivation, whereas *A. parasiticus* presented the lowest invertase production (0.8 U/ml) after 48 h of cultivation; invertase production of *A. terreus*, *A. flavus*, and *A. ochraceus* was 4.5, 6.6, and 4 U/ml, respectively (Fig. 1).

Optimization by response surface methodology for extracellular invertase production produced by *A. niger* using RSM

Statistical and mathematical analysis of multivariable data obtained from RSM is one of the most important tools to improve and optimize invertase production by *A. niger*. In the RSM, a central composite model was selected for invertase production using a 20-run experimental design with important factors such as temperature, PH, and incubation time. 20 trials matrix with three factors and three levels (−1, 0, and +1) including three replicates at the central point,

Figure 1



Invertase activity for Aspergillus strains.

Table 2 Design of different trials of the RSM for independent variables and responses by *Aspergillus niger*

Run	Factor 1 A: temperature (°C)	Factor 2 B: pH	Factor 3C: incubation time h	Activity U/ml	Actual value	Predicted value	Residual
1	(+1) 45	(+1) 9	(−1) 24	0.8	0.8000	0.4368	0.3632
2	(+1) 45	(0) 7	(0) 48	2.6	2.60	2.60	−0.0013
3	(0) 35	(−1) 5	(0) 48	6.9	6.90	5.89	1.01
4	(0) 35	(+1) 9	(0) 48	2.1	2.10	2.30	−0.1998
5	(−1) 25	(0) 7	(−1) 24	2.8	2.80	2.88	−0.0841
6	(+1) 45	(+1) 9	(+1) 72	1.5	1.50	1.37	0.1321
7	(0) 35	(+1) 9	(−1) 24	1.2	1.20	1.40	−0.2050
8	(0) 35	(−1) 5	(−1) 24	3.3	3.30	3.36	−0.0575
9	(0) 35	(0) 7	(−1) 24	3	3.00	2.87	0.1334
10	(+1) 45	(−1) 5	(−1) 24	1	1.0000	1.13	−0.1259
11	(0) 35	(−1) 5	(+1) 72	8.2	8.20	8.31	−0.1094
12	(+1) 45	(0) 7	(0) 48	2.5	2.50	2.60	−0.1013
13	(0) 35	(+1) 9	(0) 48	2	2.00	2.30	−0.2998
14	(+1) 45	(0) 7	(−1) 24	1	1.0000	1.27	−0.2668
15	(−1) 25	(0) 7	(+1) 72	7.2	7.20	6.96	0.2434
16	(0) 35	(−1) 5	(+1) 72	8	8.00	8.31	−0.3094
17	(−1) 25	(0) 7	(+1) 72	7	7.00	6.96	0.0434
18	(−1) 25	(−1) 5	(0) 48	6.5	6.50	6.91	−0.4119
19	(−1) 25	(+1) 9	(−1) 24	1	1.0000	0.7907	0.2093
20	(0) 35	(0) 7	(−1) 24	2.9	2.90	2.87	0.0334

Table 2 illustrated the independent variables with coded matrix, responses furthermore experimental and predicted values for invertase activity. The modification in the enzyme activity was observed during the twenty runs of the experiments

The alteration within the enzyme activity was owing to the different conditions in the experiment in each run, reflecting the importance of statistical optimization of fermentation condition over the traditional fermentation methodology. The maximum invertase activity was obtained after an optimized culture condition at 5 pH, 35°C-incubation temperature for 72 h with activity of 8.2 U/ml (Run11).

The determination coefficient (R^2) showed the accuracy of the model. R^2 was 0.9858, explaining 98.5% of the variability in the response could be accounted for the model, and was considered as a significant correlation. Therefore, the present value of R^2 confirms the reliability of the current model for invertase production and also exhibited a good correlation between the experiment and theoretical values.

A second-order final equation in terms of actual factors is as follows:

$$Y,\ \text{Activity}_{=} - 4.78025 + 0.289580X_1 + 0.513634X_2 + 0.251936X_3 + 0.031585X_1X_2 - 0.001571X_1X_3 - 0.017015X_2X_3 - 0.007912{X_1}^2 - 0.121351{X_2}^2 - 0.000091{X_3}^2$$

where Y represents response or invertase yield and X1, X2, and X3 is temperature, pH, and incubation time, respectively.

The model validation

The validity of the proposed model was estimated by prediction of *A. niger* invertase production for each trail of the matrix. The experimental results in Table 2 showed that the maximum observed invertase production (8.2) was very close to the predicted value (8.3) run11.

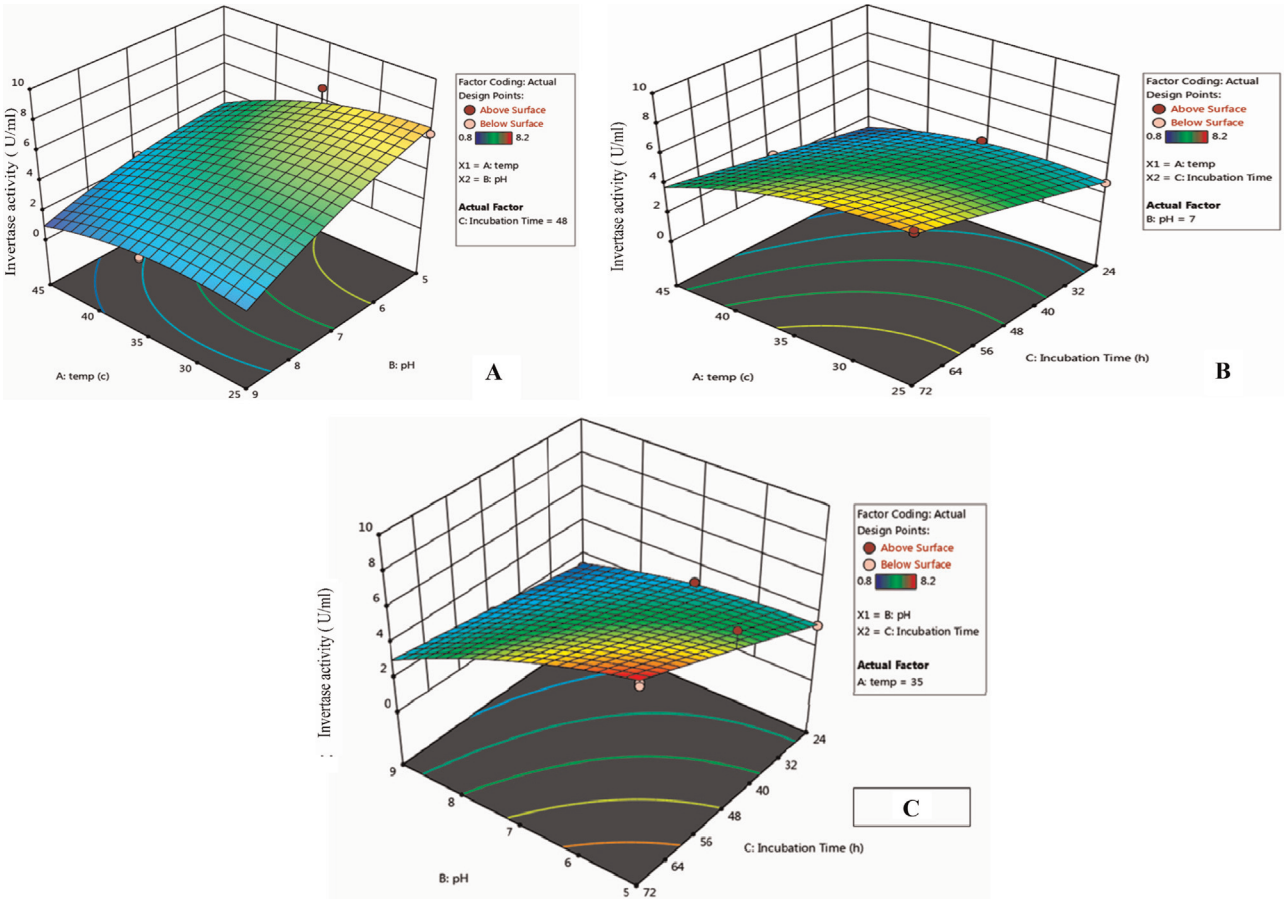
The results obtained by analysis of variance analysis for the production of invertase by *A. niger* is given in Table 3. It showed that the model was highly significant with an F value of 77.33 for invertase production and showed that it was a significant model which is evident from Fisher's '*F*' test along with a very low probability value (P model > F=0.0001). Values of 'Prob>*F*' that are less than 0.05 signify significant model, and at the same time, a relatively lower coefficient of variation (CV=7.28%) was indicated, implicating a better precision and reliability of the experiments carried out. The results obtained from this study revealed that agreement between the actual values and the predicted values and all factors have a significant response on the invertase production data, as presented in Table 3. The statistical optimization increased the biosynthesis of invertase about 1-fold of that of the basal medium (U/ml). The influence on

Table 3 Analysis of variance for invertase production *by Aspergillus niger*

Source	Sum of squares	*d.f.*	Mean square	*F* value	*P* value
Model	129.36	9	14.37	77.33	<0.0001*
A-temp	12.48	1	12.48	67.14	<0.0001
B-pH	31.03	1	31.03	166.94	<0.0001
C-incubation time	30.15	1	30.15	162.18	<0.0001
AB	1.68	1	1.68	9.06	0.0131
AC	0.9151	1	0.9151	4.92	0.0508
BC	4.29	1	4.29	23.10	0.0007
A^2	2.54	1	2.54	13.65	0.0041
B^2	0.9438	1	0.9438	5.08	0.0479
C^2	0.0105	1	0.0105	0.0566	0.8167
Residual	1.86	10	0.1859		
Lack of fit	1.80	5	0.3608	32.80	0.0008*
Pure error	0.0550	5	0.0110		
Cor total	131.22	19			

R^2=0.9731; CV=7.28%. >*F*<0.05. *Significant at prob.

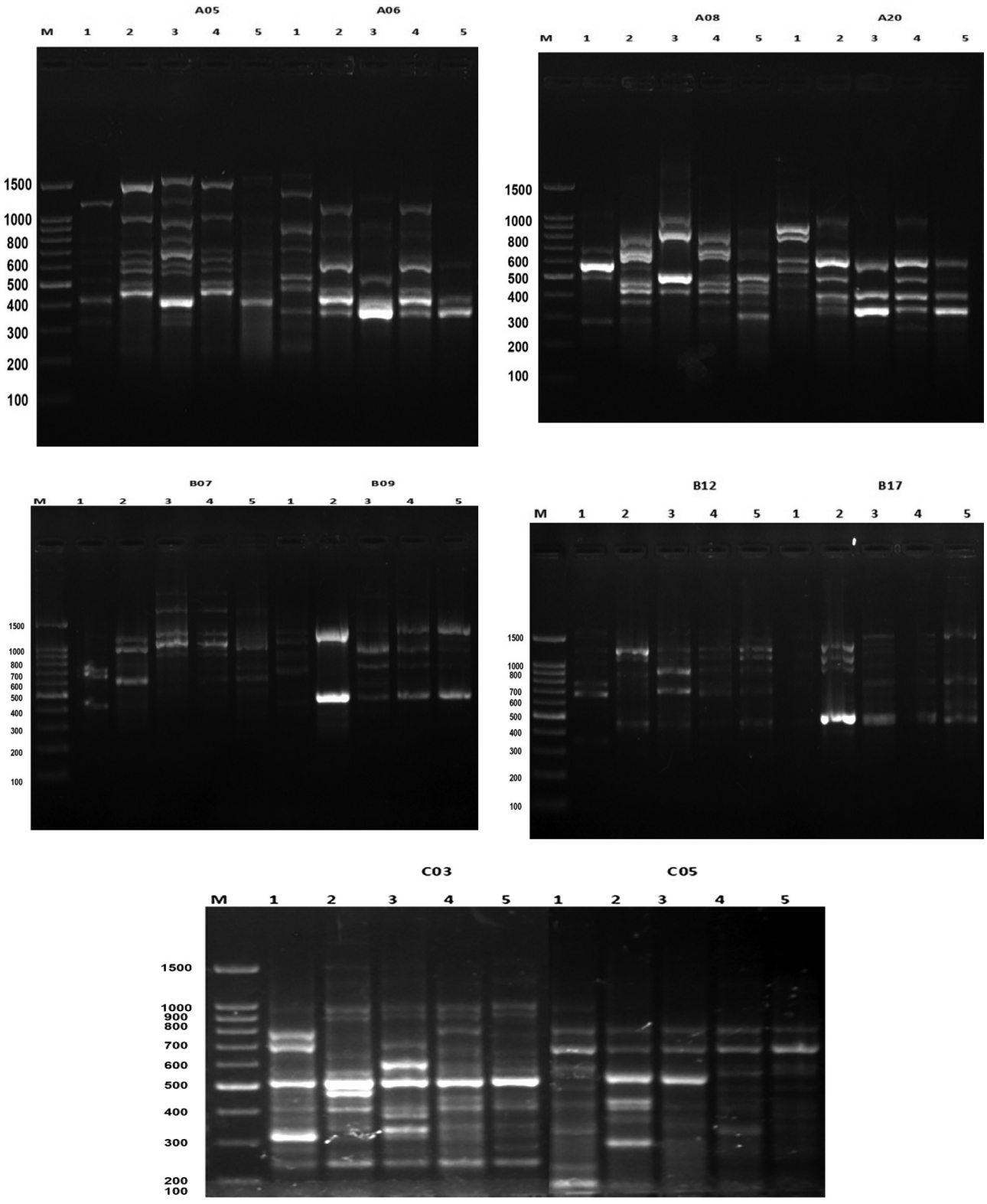
Figure 2



Response surface plot of the interaction effect of (A) temp, pH (B) temp, incubation time, and (C) pH and incubation time on invertase production by *Aspergillus niger*.

the yield of invertase imposed by the factors and reciprocity between them is represented in Fig. 2a–c. The response surface curves were designed to reveal the interaction between different variable factors and determine the optimum level of each variable for maximum response. Each figure indicated the effect of two factors, whereas the other factors were fixed at zero levels; the highest response value was detected at the result points 25°C and pH 5 for 72 h.

Figure 3



RAPD-PCR pattern produced with 10 primers. M ladder 100-bp lane (1–5) *Aspergillus* spp.

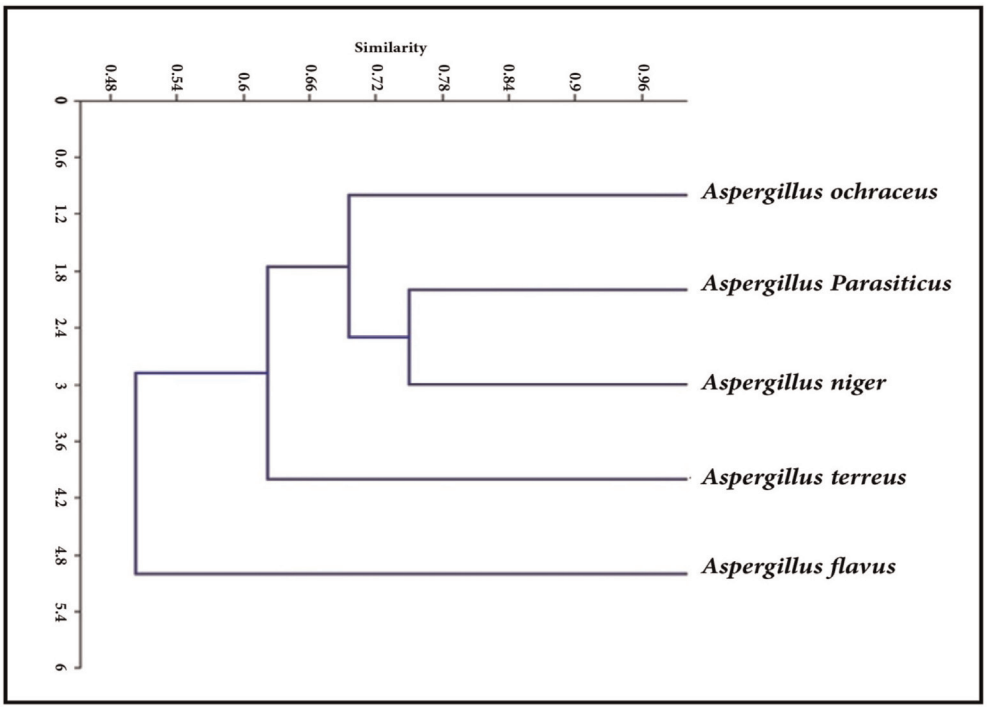
RAPD-PCR analysis

To study the genetic difference among the five *Aspergillus* spp., DNA samples were subjected to RAPD-PCR analysis using 10 primers. The pattern of these primers shows instructive and clear profile for each strain (Fig. 3) Table 4 revealed that the ten primers produced a total of 131 bands detected among the five strains. Only 70 of them were polymorphic. The percentage of polymorphism ranged from 75 to 100%. Primer OPA-08 produced the highest percentage of polymorphism, whereas primers OPB17 and OPC-03 produced the lowest

Table 4 RAPD-PCR primers names and statistical analysis

No	Name of primer	Monomorphic bands	polymorphic bands	Number of unique bands	Total bands	Polymorphism (%)	MW range (bp)	Mean of frequency
1	A-05	2	13	1	16	88	192–1654	0.6
2	A-06	1	9	6	16	94	204–1709	0.5
3	A-08	0	6	9	15	100	140–1056	0.5
4	A-20	2	5	5	12	83	209–982	0.5
5	B –07	1	10	0	11	91	331–1848	0.6
6	B-09	2	2	6	10	80	295–1294	0.5
7	B-12	2	6	2	10	80	334–1557	0.6
8	B-17	3	5	4	12	75	372–1478	0.6
9	C-03	4	9	3	16	75	232–1020	0.6
10	C-05	3	5	5	13	77	199–1027	0.5
Total		20	70	41	131	84.3		5.5
Average		2	7	4.1	13.1			0.55

Figure 4



Dendrogram showing the relationships and polymorphism between fungal strains based on RAPD-PCR analysis.

Table 5 Similarity matrix of fungal strains based on RAPD-PCR analysis

	Matrix similarity				
Isolates	1	2	3	4	5
1	1				
2	45	1			
3	52	51	1		
4	51	73	68	1	
5	52	65	67	75	1

percentage of polymorphism (75%). The RAPD-PCR profiles were generated by these primers and have multiple bands varying from 10 to 16. These primers OPA-05, OPA-06, and OPC-03 produced the maximum number of bands (16 bands), whereas the lowest two were 10 bands and generated with primers OPB-07 and OPB-09. A dendrogram was constructed after numerical analysis of RAPD-PCR data (Fig. 4), based on the similarity matrix. The results also showed the closest relationship between them. In this study, RAPD-PCR is a powerful tool to differentiate between *Aspergillus* spp.

The similarity matrix between fungal strains revealed that isolates *A. parasiticus* and *A. niger* showed a 75% similarity in Table 5. Results also showed that both *A. terreus* and *A. niger* had a similarity of 67%, followed by *A. flavus* and *A. niger* with a similarity of 52%. Lower

similarities were recorded between *A. flavus* and *A. ochraceus* (45%) and between *A. flavus* and *A. terreus* (52%).

Cluster analysis

The dendrogram was constructed using Unweighted Pair-Group Method with Arithmetical average cluster analysis to reveal the genetic relationships among five fungal isolates based on the similarity matrix between isolates. Figure 4 shows three major clusters: the first cluster included *A. flavus* isolate separate group; the second cluster (interposed group) contained *A. terreus*, divided at two subcluster *A. parasiticus* and *A. niger* isolates; and the third group contained *A. ochraceus*.

Discussion

Numerous microorganisms, especially the genus Aspergillus that possesses fructosyltransferases (invertase) with high transfructosylating activity, have been regarded as the most efficient producers of fructooligosaccharides. *A. niger* proved to be the most appropriate strain for invertase production. The productivity of the enzyme from *A. niger* was extremely high. Nguyen *et al.* [15] previously reported the production of fructooligosaccharides using β-fructofuranosidase from *A. niger* IMI303386. Mao *et al.* [16] isolated novel invertase from *A. niger* TCCC41686. Our results agree with Oyedeji *et al.* [17], who also reported that there was appreciable invertase production after 72 h (15.29±0.60 U/ml) incubation of *Aspergillus niger*. Furthermore, Veana *et al.* [18] reported that only *A. niger* GH1 among eight strains evaluated produced 8.6 U/ml invertase in less time under submerged cultivation after 48 h. The effect of improvement parameters under examination was assessed to acquire the most extreme yield of enzyme production. There are many reports for evaluation of the perfect culture conditions for production from *A. niger* using RSM method; these results agree with Ire *et al.* [19], who also used this methodology to optimize the invertase production from *A. niger* grown on low-cost agricultural wastes. Ben Mefteh *et al.* [20] used the RSM method to optimize an acidic protease produced by *Penicillium bilaiae*. RSM is gaining attention owing to its suitability in effectively aggregating optimal conditions for multivariable schemes [5]. This technique was utilized in the optimization of environmental conditions necessary for the production of invertase. Applying RSM, at the same time, the determination of the main and interaction effects of different environmental factors on invertase production was permitted. Sivakumar *et al.* [21] used the RSM technique to optimize keratinase production by *Bacillus cereus* and concluded that RSM can improve yield as well as the cost of enzyme production. The use of RSM in optimizing bioprocess techniques has been heralded as an effective way of determining the most suitable parameters for the production process. Senthivelan *et al.* [12] also used RSM to improve and optimize the activity of laccase enzyme. The maximum activity (7.9 U/ml) was detected after optimization for 5 days and in cultivation at 32°C by the potential white-rot fungus *Penicillium chrysogenum*. This technique was more accurate than the classical method (6.0 U/ml). RAPD-PCR technique was used to study the genetic diversity and relationships between *Aspergillus* strains. RAPD-PCR has been successfully employed in phylogenetic and genetic diversity studies, as it is simple, inexpensive, and has high reproducible profiles. These results are in agreement with Hashoosh *et al.* [22] who also used RAPD-PCR to differentiate between twenty-four isolates of *A. niger* isolated from different sources (soil, seeds, powdered milk, and factory wastewater). Similar observations were reported by Adss *et al.* [23], who used RAPD and ISSR to differentiate between seven isolates of *A. solani* and their pathogenic capability.

Conclusion

In the current investigation, some filamentous fungi were screened for invertase production. *A. niger* was the most promising strain for enzyme production. RSM seems to the prospective technique for optimization of microbial enzymes. The current study points to the successful optimization of invertase production after a combination between various factors such as 35°C temperature, pH 5, and incubation time for 72 h. The obtained data from this investigation have demonstrated a significant increase in extracellular invertase production by *A. niger* through RSM. Invertase production was the result of a synergistic combination of effective parameter interactions. The optimized conditions for the production of invertase enzyme as reported in this study can be of several advantages, with a wide range of industrial applications such as the food industry. The use of fungi for the invertase production seems to be a promising candidate, with a eukaryotic origin. RAPD markers have the potential technique for differentiation between *Aspergillus* spp.

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Conflicts of interest
There are no conflicts of interest.

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