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Second Generation Biofuel Production from *Moringa oleifera* Pod Husks Utilizing Cellulases of A New Decaying Fungus; *Cladosporium halotolerans* MDP OP903200

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DEPLETION of fossil fuels and saving global food resources have represented one of the main future challenges. Although first-generation bioethanol production from sugary food materials has provided a sustainable green biofuel source, it has resulted in a "fuel-food" conflict. Thus, second-generation bioethanol production from nonedible lignocellulosic materials has gained great attention. This study aimed to exploit *Moringa oleifera* pod husks "one of the least studied agricultural wastes" for 2nd generation bioethanol production utilizing a green and cost-effective process. A new isolate "*Cladosporium halotolerans* MDP OP903200" has been isolated from decaying Moringa dry pods. Obvious structural changes stimulated by this fungus on dry pods have been observed due to its cellulolytic activity. Its cellulase productivity has been optimized by employing response surface methodology. The best cellulase activity was recorded when incubation temperature, inoculum size, and incubation period values were shifted to 22°C, one (8mm) fungal disc and 13 days respectively. The best temperature and pH values for its cellulolytic reaction were 50°C and pH 5. Upon the completion of the optimization study, a 4.6-fold increase in cellulase activity has been achieved compared to the un-optimized conditions. Good activity for the investigated cellulase has been recorded under the low water activity conditions which reflects its promising applicability for bioethanol production. Direct saccharification of Moringa dry pod powder by the investigated enzyme without any pretreatment process has yielded an increase in the Moringa pod simple sugar content from 93.2 to 330.6mg/gds and yielded 36.06g/L bioethanol production upon the fermentation by *Saccharomyces cerevisiae*.

Keywords: *Cladosporium halotolerans* cellulose, *M. oleifera* dry pod husks, Saccharification, Second-generation bioethanol, Response surface methodology optimization.

Introduction

Due to the rising global population and the increasing fossil fuel demands, a great global energy issue has been generated. In addition, the combustion of such fossil fuels has resulted in many environmental and health issues due to the pollutants that are liberated during this process (Pant et al., 2023). Hence, there is a growing interest in researching other alternative green and renewable energy sources (Makkawi et al., 2023).

Bioethanol is one of the most prevalent eco-friendly biofuels that has been produced and effectively used as an alternative fuel in many countries. Presently, commercial bioethanol production depends on the fermentation of edible food crops such as sugarcane, sweet potato, barley, corn, and wheat (Hassan et al., 2021; Tusher et al., 2022). This first-generation (1G) bioethanol production process has resulted in a "food vs fuel" conflict and generated another question; "How to maintain a balance between providing new and renewable energy supply and securing global food resources?" Thus, recently the production systems have focused on the use of non-consumable

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food materials such as lignocellulosic biomass produced as agriculture wastes rather than these edible sources (Hassan et al., 2021; Valladares-Diestra et al., 2022). Ethanol produced from these non-edible feedstock and agricultural wastes (cellulosic ethanol) is considered as a second-generation biofuel (Kumar et al., 2022; Valladares-Diestra et al., 2022).

Moringa oleifera (family: Moringaceae) is a promising medicinal plant that can be considered as a 'miracle tree'. It represents a source for many valuable pharmacological and health-promoting compounds; moreover, its derived meals are with high nutritional value (Raman et al., 2018; Giuberti et al., 2021; Kapse & Samadder, 2021). As a result, cultivation of Moringa tree has been greatly encouraged in developing regions to increase food security and reduce malnutrition issues specially for infants and child-bearing age women (Raman et al., 2018). Recently, this tree's cultivation has been expanded around the world as it is fast-growing (Mirhashemi et al., 2018; Giuberti et al., 2021), ever green, resistant to drought (Mirhashemi et al., 2018; Kapse & Samadder, 2021), and tolerant to poor soils and a wide range of soil pH (5.0–9.0) (Mirhashemi et al., 2018).

Although all parts of this tree can be exploited in varying applications (Giuberti et al., 2021), Moringa seed pods are still considered as one of Moringa cultivation's useless wastes (Shirani et al., 2018). Compared to other parts of *Moringa oleifera* tree, Moringa seed pods have received little attention concerning their applicability (Oladele et al., 2022). Within nearly three years a tree can yield 400-600 pods annually while a mature tree can produce up to 1,600 pods (Horn et al., 2022). These pods are mainly composed of cellulose, hemicellulose, and lignin (Lehman et al., 2018). It has been estimated that each one kilogram of pods contains 512±4.81g cellulose (Melesse & Berihun, 2013). Thus, it can be considered as a promising inexpensive and renewable feedstock for bioethanol production upon saccharification of its cellulose content. However, few studies have been performed on the utilization of such promising cellulosic feedstock for bioethanol production. The first exploratory study on this concern was published in 2013; up to 2017, only three published studies have evaluated ethanol production using Moringa pod husks and seeds husks (Montaño et al., 2017). Most of these studies have examined the saccharification of Moringa pod husks chemically, and only one study has estimated its saccharification enzymatically after an acid/temperature pre-treatment process.

Saccharification of cellulose can be maintained by the conventional chemical hydrolysis method. However, enzymatic saccharification by cellulase became the most effective strategy used in the conversion of cellulosic materials into reducing sugars due to its cost-effectiveness, consistency, ease of process optimization, low energy requirement, and less resulting pollution issues (Zhao et al., 2021; Rajesh & Gummadi, 2022).

Although cellulases can be produced from animal and plant sources, microbial cellulase has gained great interest as it shows higher stability, lower production cost, and faster and easier production methods (Ali et al., 2022). Several species of bacteria and fungi can produce cellulases (Rajesh & Gummadi, 2022). Due to the increasing demand for cellulases, it becomes necessary to explore new cellulase-producing microbial isolates from different ecological habitats (Bhardwaj et al., 2021) to produce cellulase with promising characteristics.

Vázquez-Montoya et al. (2020) have isolated and purified 48 microbial isolates from stem and leave samples collected from *M. oleifera* trees growing at different sites in the State of Sinaloa, Mexico. Among them 42 isolates have shown cellulolytic activities, and the best activities were recorded for 3 fungal isolates: *Penicillium funiculosum*, *Fusarium verticillioides* and *Cladosporium cladosporioides*.

Hence, this study aims to saccharify *Moringa oleifera* seed pods, one of the least studied cellulose-rich agricultural wastes, utilizing cellulase from a new Moringa pod decaying fungal isolate. Utilization of such inedible cellulosic feedstock for the production of second-generation biofuel can limit the dependence on edible sugar-containing food materials for ethanol production in an attempt to save food resources and lower the cost of the production process.

Materials and Methods

Microorganism

The investigated fungus was isolated from decaying *Moringa oleifera* dry pod samples collected from Giza, Egypt in January 2022

(30°3'45"N 31°11'24"E). Surface decaying features of dry pod samples were examined using stereo microscope (KYOWA Tokyo No.981826). Isolation of decaying fungus was carried out on MGYP Agar (Malt extract; 3.0, Yeast extract; 3.0, Peptone; 5.0, Glucose; 10.0, Agar; 20.0g/L: pH=6.2, Wickerham, 1951) supplied with rose bengal for suppression of bacterial growth. Sequencing of ITS region has been utilized for molecular identification of this fungal isolate by using the universal primers ITS1 (5' - TCCGTAGGTGAA CCTGCGG - 3') and ITS4 (5' - TCCTCCGCTTATTGATATGC -3'). DNA extraction, PCR reaction and sequencing of amplicon have been carried out at Solgent Company, Daejeon, South Korea. The DNA was extracted by SolGent purification bead, and the PCR reaction is carried out by BigDye® Terminator v3.1 cycle sequencing Kit using ABI 9700 Thermocycler (USA). The reaction mixture consisted of 2µL of premix, 6µL buffer, 50 to 400ng from template, and 3:5pMol primer. The PCR reaction was performed according to the following conditions: Denaturation temperature is 95°C for 15min, annealing temperature is 50°C for 40sec, and extension temperature is 72°C for 1min and for 5min at the final extension step. The DNA analysis was carried out by ABI 3730XL DNA Analyzer. The obtained DNA sequence was analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website which also has been utilized in phylogenetic analysis of this sequence. Then, the obtained sequence was uploaded to the GenBank database to obtain the accession number of the fungal isolate (sequence is accessible at https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_2359483998.

Assessment of cellulolytic activity

Cellulosic material

Undecayed *Moringa oleifera* dry pod samples were collected from the aforementioned site. Seeds were deshelled manually to obtain empty Moringa Dry pods that were dried at 60°C for 5 days then powdered using a blender to be used as the cellulosic material in this study.

Submerged fermentation

Two fungal discs (disc diameter =0.8cm) were inoculated to 250mL conical flasks containing 50mL broth basal medium (KH_2PO_4; 4.0, Na_2HPO_4; 4.0, Tryptone; 2.0, $MgSO_4.7H_2O$; 0.2, $CaCl_2.2H_2O$; 0.001, $FeSO_4.7H_2O$; 0.004g/L; pH=7 Behera et al., 2017) supplemented with 1% dry pod powder as a cellulosic material for cellulase induction. Flasks were incubated at 25°C for 7 days under static conditions. The growing mycelium was separated from the culture medium via filtration using Whatman filter paper no.1 and the obtained filtrate was used to estimate the crude enzyme activity.

Cellulase assay

Cellulase activity was estimated using the dinitrosalicylic acid (DNS) method described by Miller (1959). The evaluation of hydrolysis of Carboxymethyl Cellulose (CMC) by cellulases was evaluated by estimating the amount of reducing sugars (glucose) released from the enzymatic reaction with the help of a glucose standard curve. In this method, 0.5mL of crude enzyme was mixed with 0.5 ml of 1% CMC solution (prepared with Citrate buffer; 0.1M, pH 6.0). The reaction mixture was incubated at 55°C for 30min; then, the reaction was stopped by adding 3 ml of DNS reagent and boiling for 10min. The mixture was immediately cooled to room temperature and the absorbance was measured at 540nm to estimate the amount of released reducing sugars utilizing a pre-established glucose standard curve. One Unit Enzyme activity (U) has been defined as the amount of enzyme that releases 1µmol of reducing sugar (glucose)/min. Control measurements (i.e., reaction mixture without enzyme and reaction mixture without CMC substrate) were subtracted from assay's readings, and the test was performed in a replicated manner.

Estimation of fungal growth

Fungal growth was evaluated by measuring the dry biomass weight. The filtered mycelia were washed with distilled water and dried at 60°C in the oven until obtaining successive equal weights.

Saccharification of Moringa dry pod powder

The amount of reducing sugars released from hydrolysis of Moringa dry pod cellulose using the investigated cellulase has been estimated. In this method, 0.5mL of crude enzyme was mixed with 0.5mL of 1% dry pod powder solution (prepared with Citrate buffer; 0.1M, pH 6.0) and incubated at 55°C. The reaction was terminated after 30min, and the amount of released reducing sugars was estimated using the aforementioned DNS method.

Optimization of cellulase production by the investigated fungal isolate using response

surface methodology

Box-Behnken design (BBD)

Three important fermentation parameters were subjected for optimization study employing a response surface methodology design "Box-Behnken design" to enhance cellulase production by the investigated fungal isolate. Incubation temperature, inoculum size and incubation period were studied at three levels; low (-1), intermediate (0), and high (+1), as shown in Table 1. The design has generated fifteen trials, as illustrated in Table 2, including three replicated central points for error control. In this study, cellulase activity was the monitored response. Results were fitted with the following second-order polynomial equation form:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2,$$

Y, β_0, β_i, β_{ij}, β_{ii}, $X_i X_j$ refers to the predicted response, the intercept term, the linear coefficient, the quadratic coefficient, the interaction coefficient, and the independent variables respectively (Jose et al., 2013). The obtained results were subjected to regression analysis utilizing the 'Design Expert' software package (Version 7.0). The accuracy of the generated polynomial model equation was evaluated by estimating R^2 and adjusted R^2 values. The three-dimensional response surface plots were generated to visualize the main effect and factor-factor interaction. Numerical optimization analysis was employed to estimate the recommended optimized level of each variable for a maximum response.

Experimental validation

One of the solutions predicted by the numerical optimization analysis tool for maximum cellulase productivity was tested experimentally and considered as a checkpoint for model validation and for the calculation of the deviation percent between the predicted and the actually obtained values.

TABLE 1. Levels of tested factors used in cellulase production optimization employing Box-Behnken design

Factor	Unit	Low level (-1)	High level (+1)	Intermediate level (0)
Incupation temperature	°C	15	35	25
Inoculum size	Fungal discs "0.8cm"	1	5	3
Incubation period	days	3	13	8

TABLE 2. Box-Behnken design matrix for optimizing cellulase production by *Cladosporium halotolerans* (OP903200)

Trail no.	Incupation temperature (°C)	Inoculum size (Fungal discs "0.8cm")	Incubation period (Days)	Response (Cellulase activity U/mL)
1	35.00	3.00	3.00	0.0466623
2	25.00	1.00	3.00	0.0605784
3	15.00	5.00	8.00	0.0684062
4	15.00	3.00	3.00	0.0528593
5	25.00	3.00	8.00	0.0551424
6	25.00	1.00	13.00	0.112764
7	25.00	5.00	13.00	0.0967819
8	35.00	1.00	8.00	0.0419874
9	15.00	1.00	8.00	0.06384
10	15.00	3.00	13.00	0.0884105
11	35.00	5.00	8.00	0.036334
12	25.00	3.00	8.00	0.0563383
13	35.00	3.00	13.00	0.0381822
14	25.00	5.00	3.00	0.0621005
15	25.00	3.00	8.00	0.061122

Optimization of cellulolytic activity

After the optimization of cellulase production by the investigated fungal isolate, cellulolytic activity was further optimized by estimating the best reaction temperature and pH values.

Reaction temperature

The reaction mixture was incubated at different temperature values (30, 40, 50, 60, 70, 80°C) for 30min and cellulase activity was evaluated by estimating CMC hydrolysis using the aforementioned dinitrosalicylic acid (DNS) method.

Reaction pH

Cellulase activity at 3.0 - 8.0 pH range was investigated using suitable buffer systems in a concentration of 0.1M (pH 3, 4, 5, 6; citrate buffer & 7, 8; phosphate buffer). The reaction mixture was incubated for 30min at the estimated optimal temperature and cellulase activity was evaluated as described previously.

Evaluation of cellulolytic activity under low water activity

Cellulase activity at different sodium chloride concentrations (0, 2.5, 5 and 7.5%) was estimated to evaluate its applicability under low water activity conditions. The cellulase activity was evaluated as indicated earlier.

Bioethanol production using Moringa dry pod powder

The use of *Moringa oleifera* dry pod powder as a substrate for bioethanol production utilizing the produced cellulase was evaluated.

Enzymatic saccharification

Moringa oleifera dry pod powder was hydrolyzed utilizing the investigated crude cellulase for the release of reducing sugars. Equal volumes of crude enzyme and sterilized dry pod powder solution (1.5%, pH 5) were mixed and incubated at 50°C for 96h. The enzymatically hydrolyzed sample was estimated for total reducing sugar content.

Fermentation

Saccharified *Moringa oleifera* dry pod powder solution was inoculated with 1% (w/v) *Saccharomyces cerevisiae* "Commercial dry yeast" and incubated at 30°C for 6 days. Ethanol production was estimated after 24h and 6 day intervals using the chromic acid method (Caputi et al., 1968). 0.1mL sample was diluted to 5mL with distilled water and added to 5mL chromic acid reagent (34g of potassium dichromate in 500mL distilled water with addition of 325mL of conc. H_2SO_4 and making the volume up to 1000mL). The reaction mixture was incubated at 60°C for 20min and then cooled and absorbance was measured at 584nm. Ethanol concentration was calculated from a pre-established standard curve maintained using absolute ethanol.

Statistical analysis

Results are presented as a mean value of replicates with standard error (±Std. Error). Statistical analysis in the experimental design optimization study was performed by "Design Expert" software package Version 7.0. The variable is considered significant if the estimated P-value < 0.05. One-way analysis of variance ANOVA was performed by Minitab statistical package version 17.1.0 to compare groups (more than 2 groups) followed by pairwise comparison analysis to visualize the grouping information using Tukey method at 95% confidence.

Results

Isolation and identification of fungal isolate

Moringa oleifera dry pods showing spots of superficial fungal growth were used for the isolation of the investigated cellulose decaying fungal isolate. Examination of these pod samples using stereo microscope showed obvious surface structural changes compared to undecayed "normal" samples. Dry pod samples with superficial fungal growth spots showed loss of surface consistency, browning, increase in dryness, and appearance of multiple cracks and localized fungal growth regions as illustrated in Fig. 1.

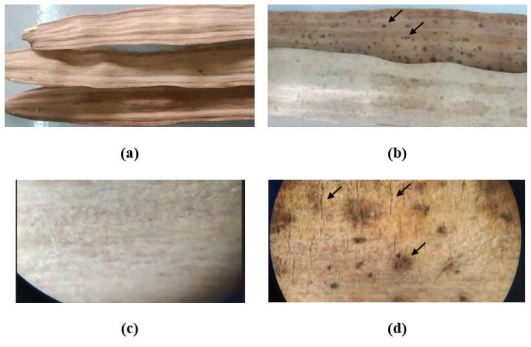


Fig. 1. Collected *Moringa oleifera* dry pods (a); normal, (b); with localized fungal growth. (c) surface feature of normal pods under

stereo microscope (d) surface features of pods with localized fungal growth under stereo microscope showing appearance of decaying features; cracks, loss of surface consistency, dryness, browning and lesions of fungal growth

Upon isolation process, a cellulase-producing fungal isolate has been isolated from these decaying *Moringa oleifera* dry pods as shown in Fig. 2. Based on the ITS sequence analysis, this isolate revealed 99.8% similarity with several *Cladosporium halotolerans* isolates. Moreover, phylogenetic tree analysis showed this isolate among the clusters of *Cladosporium halotolerans* on one clade (Fig. 3) suggesting it as a new *Cladosporium halotolerans* isolate. Depending on these molecular sequencing results and its growth and morphological characteristics, this isolate was identified as *Cladosporium halotolerans* and submitted in GenBank as *Cladosporium halotolerans* (Isolate MDP) and released under the accession number OP903200.

Assessment of cellulolytic activity

Cellulase production by *Cladosporium halotolerans* MDP OP903200 was estimated by the dinitrosalicylic acid (DNS) method. The addition of Moringa dry pod powder to the growth medium was found to efficiently induce cellulase production by *Cladosporium halotolerans* OP903200 (0.035 ± 0.005U/mL). The activity of this enzyme released 83.93 ± 15.28mg reducing sugars from one gram of Moringa dry pod powder in 30 minutes as illustrated in Table 3.

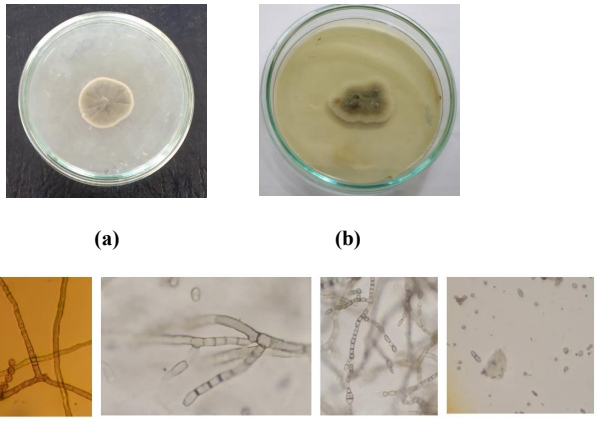
Optimization of cellulase production using response surface methodology

Box-Behnken design (BBD)

The statistical analysis of the obtained Box-Behnken design results revealed that the model is significant where the model *p* value is 0.0236, and *F*-value is 6.87, and the Lack of Fit is not significant (Lack of Fit *F*-value equals 15.65 as shown in Table 4. The model correlation coefficient value (R^2) equals 0.925, and the adjusted R^2 equals 0.800 (Table 5). That reveals the presence of a good correlation and confirms the significance of the model. It was found that A, C, A^2 and C^2 are significant model terms according to ANOVA analysis of results where their P< 0.05. The model regression analysis predicted the second-order polynomial equation as follows:

$$\text{Cellulase activity (U/mL)} = 0.058 - 0.014\,\mathbf{A} - 1.943\text{E-}003\,\mathbf{B} + 0.014\,\mathbf{C} - 2.555\text{E-}003\,\mathbf{AB} - 0.011\,\mathbf{AC} - 4.376\text{E-}003\,\mathbf{BC} - 0.016\,\mathbf{A^2} + 0.011\,\mathbf{B^2} + 0.015\,\mathbf{C^2}$$

where, A, B, and C refer to the coded factors of incubation temperature, inoculum size and incubation period respectively. Perturbation and the three-dimensional response plots have been displayed to visualize the main effect of the tested factors and the interaction among them. The main effect of tested factors on cellulase productivity was illustrated via perturbation plot as indicated in Fig. 4. It was observed that the incubation period has the major effect followed by incubation temperature where the increase in the incubation period and the decrease in the incubation temperature can lead to a higher cellulase production by *Cladosporium halotolerans* (OP903200).



(a) **(b)**

(c)

Fig. 2. *Cladosporium halotolerans* MDP OP903200 (a); On PDA medium, (b); On MEA supplied with 5% NaCl, (c); Under light microscope

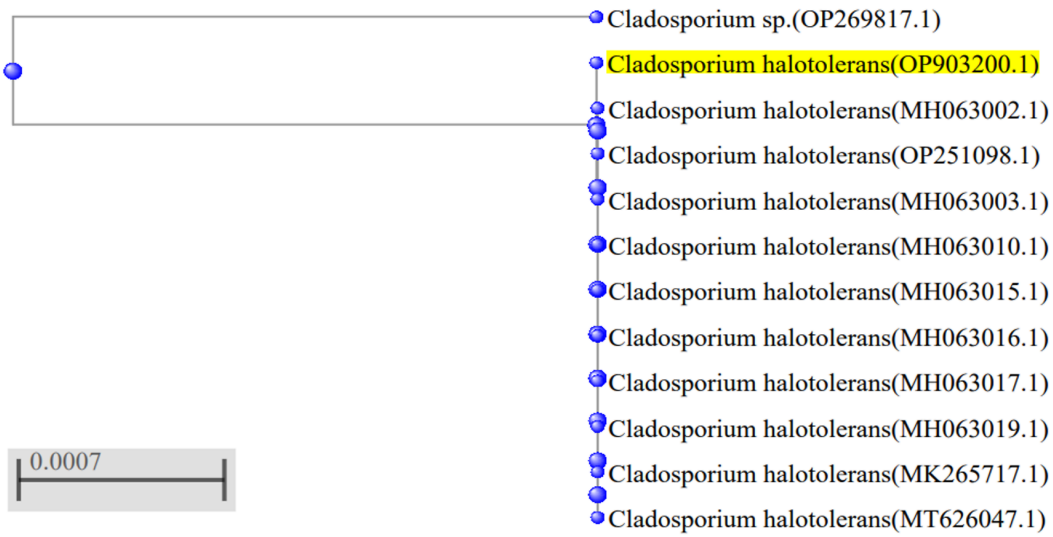


Fig. 3. Phylogenetic tree analysis showing the relationship between *Cladosporium halotolerans* (OP903200) and other closely related sequences using NCBI GenBank references taxa

TABLE 3. Cellulolytic activity of *Cladosporium halotolerans* (OP903200)

Growth dry weight (g/L)	Crude cellulase activity (U/mL)	Released reducing sugars (mg/gram dry substrate)
3.74 ± 0.88	0.035 ± 0.005	83.93 ± 15.28

Mean value followed by ± its standard error value.

TABLE 4. ANOVA statistical results of employing BBD showing the effect of tested factors on cellulase production

Variable	Effect (Coefficient)	Standard error	P value	Significance
A	-0.014	5.716E-003	0.0110	Significant
B	-1.943E-003	3.500E-003	0.6027	Non-Significant
C	0.014	3.500E-003	0.0096	Significant
AB	-2.555E-003	3.500E-003	0.6278	Non-Significant
AC	-0.011	4.950E-003	0.0768	Non-significant
BC	-4.376E-003	4.950E-003	0.4172	Non-Significant
A^2	-0.016	4.950E-003	0.0285	Significant
B^2	0.011	5.152E-003	0.0898	Non-Significant
C^2	0.015	5.152E-003	0.0357	Significant
Model	0.058	5.716E-003	0.0236	Significant

A= incubation temperature, B= inoculum size, C= incubation period.

TABLE 5. Regression Statistics for BBD

Model R^2	0.925
Model adjusted R^2	0.800

The 3D response plots (see Figs. 5-7) were obtained by displaying the effect of two factors and keeping the third factor at its zero coded level (intermediate value) to visualize both the main effect of factors and the interactive behavior between them. The plots revealed that there was no significant mutual interaction between the tested factors. The levels of tested factors that can give the highest cellulase productivity were estimated using Design Expert's numerical optimization tool.

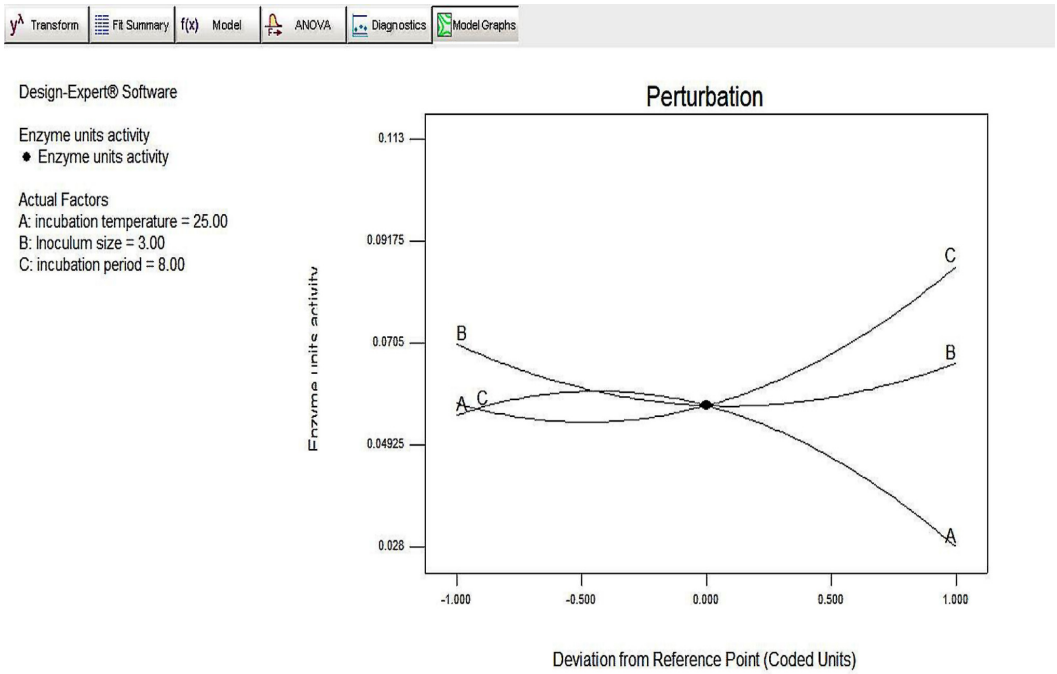


Fig. 4. Perturbation plot illustrating the main effect of A= incubation temperature, B= inoculum size, C= incubation period on cellulase production

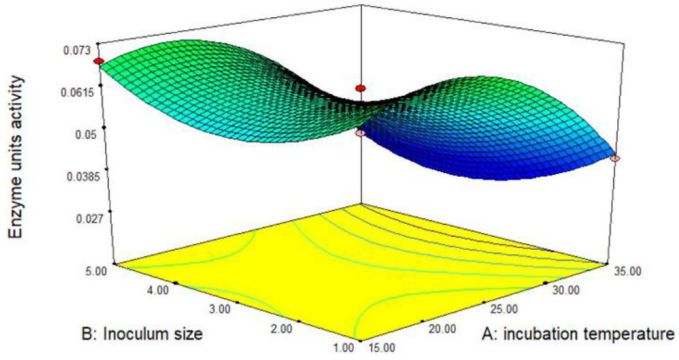


Fig. 5. 3-D. Response surface plot revealing the interaction between incubation temperature and inoculum size and its effect on cellulase production

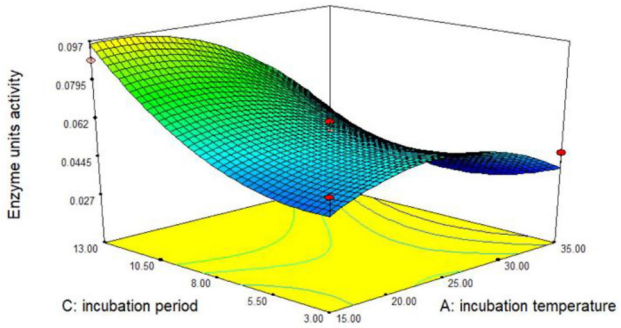


Fig.6. 3-D. Response surface plot revealing the interaction between incubation temperature and incubation period and its effect on cellulase production

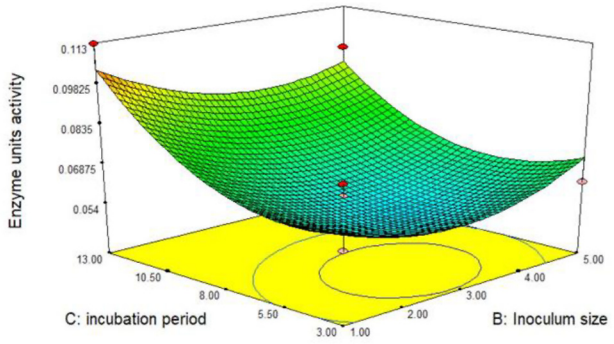


Fig.7. 3-D. Response surface plot revealing the interaction between inoculum size and incubation period and its effect on cellulase production

Experimental validation

One of the solutions predicted by the Design Expert's numerical optimization tool for the highest cellulase production by *Cladosporium halotolerans* (OP903200) was selected (Fig. 8) and tested experimentally to check the accuracy of the employed model's results for model validation. The approximated values of the recommended levels have been experimentally tested and the predicted cellulase productivity was compared with the actually obtained one. It was found that the employed solution has yielded 0.115 ± 0.00U/mL cellulase productivity giving a 7.96% deviation from the predicted productivity (0.106U/mL) which represents an acceptable deviation percent and recommends the validation of the employed model as illustrated in Table 6.

Optimization of cellulolytic activity

The activity of the investigated cellulase enzyme was monitored under different temperatures and pH values. The best activity was detected under 50°C and pH 5 (Fig. 9).

By the completion of optimization study, the cellulase activity was found to increase from 0.035U/mL to 0.162U/mL (Fig. 10).

Evaluation of halotolerance behavior of cellulase enzyme

The halotolerance behavior of cellulase enzyme and maintenance of its activity under low water activity and high ionic strength conditions was evaluated by estimating its activity in the presence of different NaCl concentrations. It has been found that, the catalytic activity of *Cladosporium halotolerans* (OP903200)'s cellulase is significantly maintained in the increased ionic strength as illustrated in Fig. 11.

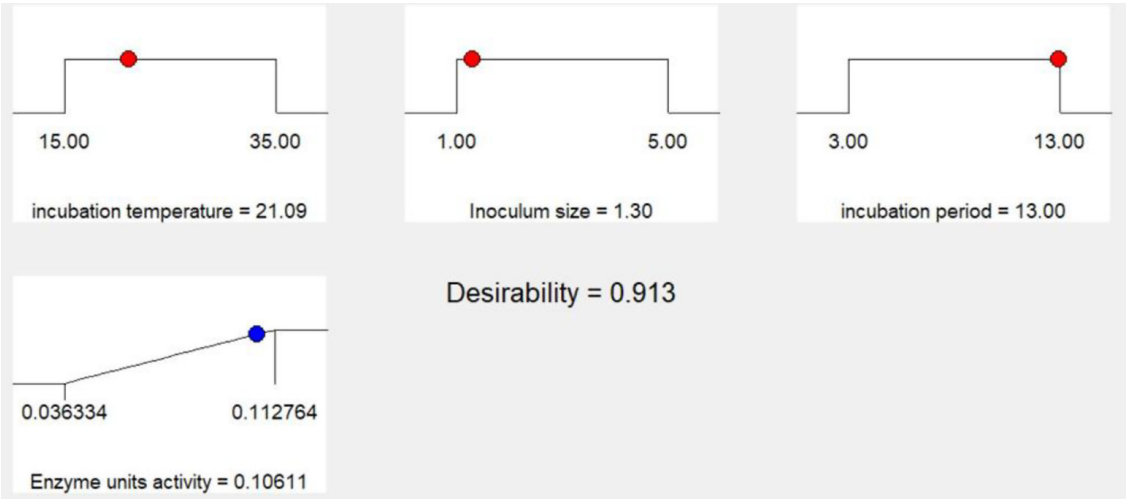
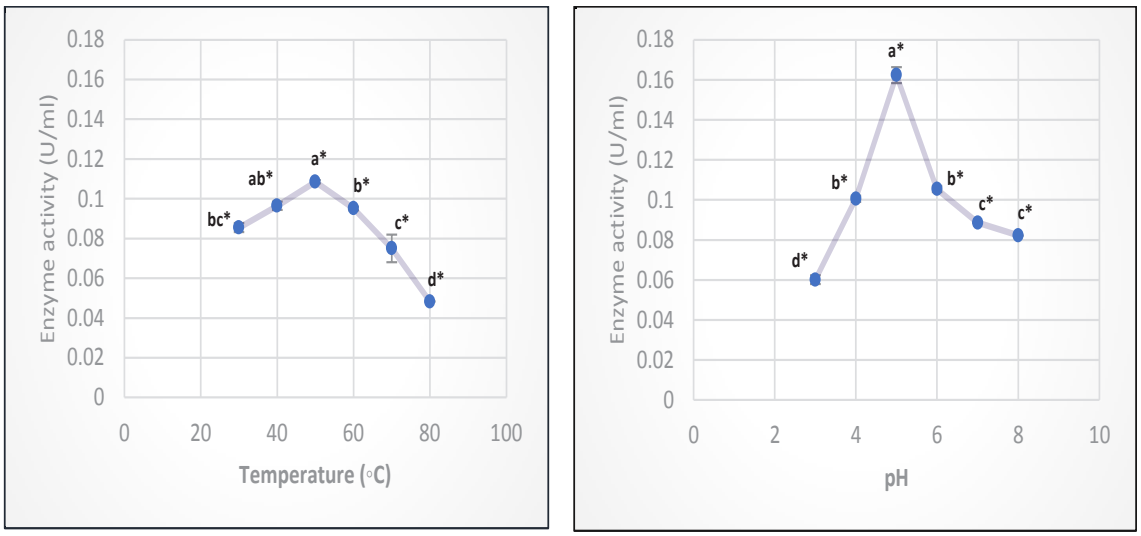


Fig. 8. Selected solution predicted by BBD numerical optimization tool for the highest cellulase production

TABLE 6. Results of model validation (predicted versus actual values to maximize cellulase production)

Method	Variable level			Cellulase activity (U/mL)	Deviation
	Incubation temperature (°C)	Inoculum size (Fungal discs "0.8cm")	Incubation period (days)		
Prediction of Design Expert's model	21.09	1.30	13	0.1061	7.96 %
Experimental value (Tested/Actual)	22	1.00	13	0.1150	



(a) (b)

Fig. 9. Effect of temperature (a) and pH (b) on crude cellulase activity [mean values are with their standard error bars, * means with different letters are with significant differences between them]

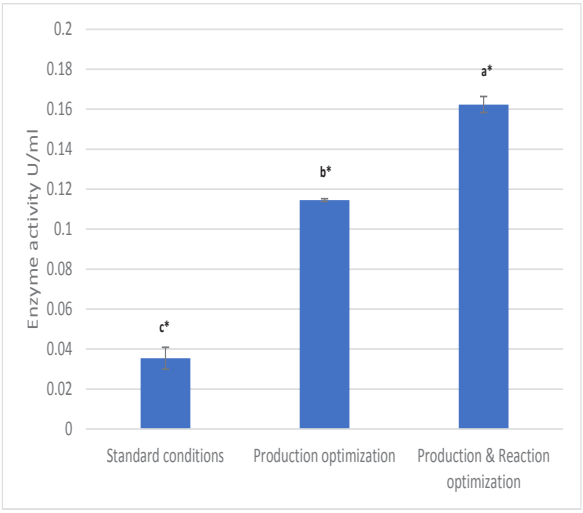


Fig. 10. Effectiveness of optimization study in enhancement of *Cladosporium halotolerans* (OP903200) cellulolytic activity [mean values are with their standard error bars, * means with different letters are with significant differences between them]

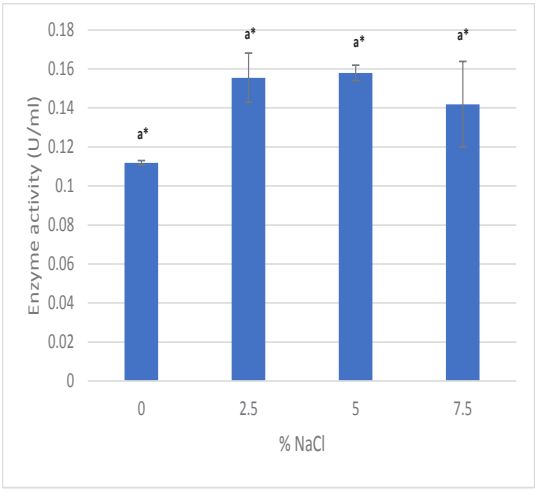


Fig. 11. Activity of *Cladosporium halotolerans* (OP903200)'s cellulase under different NaCl concentrations [* mean values are with their standard error bars and means with the same letters are not different statistically]

Bioethanol production using Moringa dry pod powder

Moringa dry pod powder was saccharified for reducing sugars production using a cellulase enzyme produced by *Cladosporium halotolerans* (OP903200). Saccharification was carried out under the pre-estimated optimal temperature and pH values for 96 hours. This step has increased the total reducing sugars content from 93.2 ±0.0mg to 230.3 ±5.3mg per gram of Moringa dry pod powder which is further increased during the following stages to 330.6 ±14.0mg/g. Upon inoculation of *Saccharomyces cerevisiae* to the saccharified sample, bioethanol production was tracked. It has been found that ethanol production has reached a concentration of 20.14 ±0.32g/L after 24h and increased to 36.06 ±0.0g/L after 6 days of fermentation, as shown in Fig. 12.

Discussion

Moringa oleifera dry pod samples showing structural decaying features associated with localized fungal growth lesions were used for isolation of a cellulose decaying *Cladosporium halotolerans* isolate. The identification of this isolate was performed by sequencing its ITS region and confirmed by studying its microscopic and cultural characteristics which were found to fit with the morphological features of *Cladosporium halotolerans* described by Zalar et al. (2007) and Kobayashi et al. (2012). Hence, this isolate was identified as *Cladosporium halotolerans* and submitted to GenBank as *Cladosporium halotolerans* MDP isolate and released under OP903200.1 accession number.

Structure decaying features associated with the growth of this fungus on *Moringa oleifera* dry pods were an indicative sign of its cellulolytic activity. Cracks and high dryness of these decaying pods represent an initial sign of the productivity of a potent cellulase that retains good activity under very low water activity. Due to data scarcity about cellulase production by *Cladosporium halotolerans*, this study concerned with the evaluation of its cellulolytic activities and the applicability of its cellulase in the saccharification of Moringa dry pod for bioethanol production.

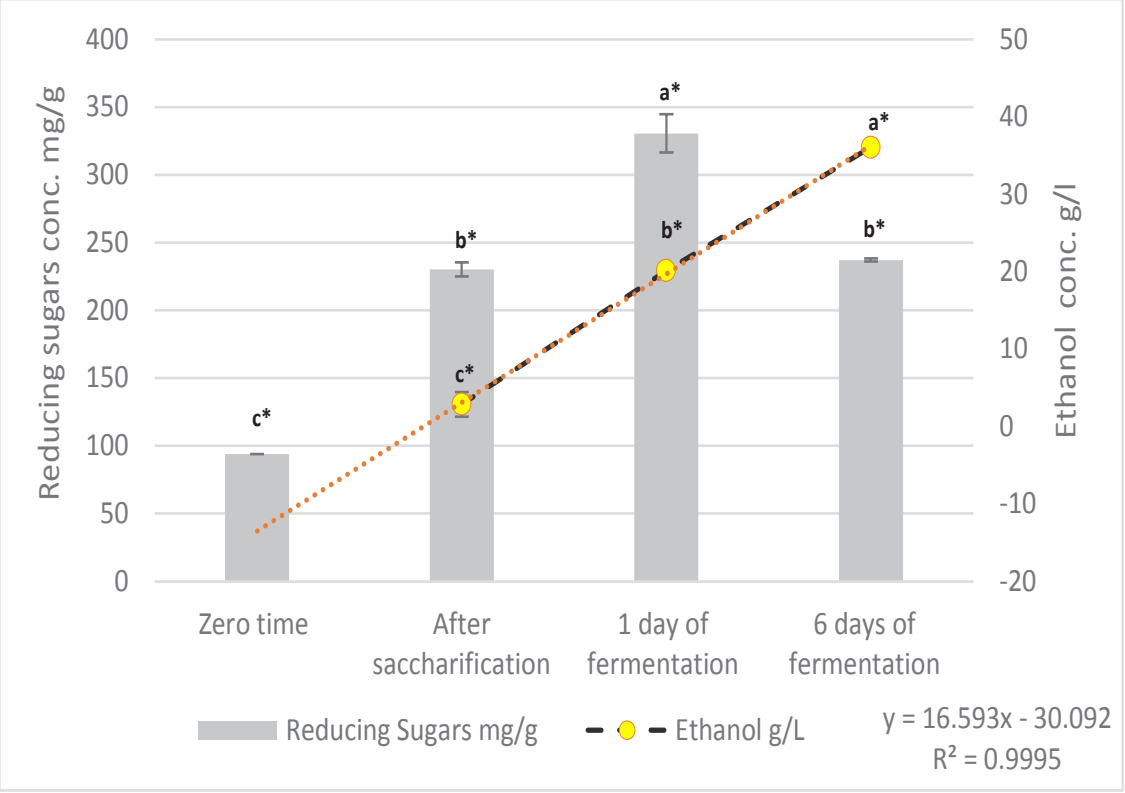


Fig. 12. Tracking of reducing sugars' release and bioethanol production using Moringa dry pod powder [* mean values are with their standard error bars, and means with the same letters are not different statistically]

The evaluation of cellulase production by *Cladosporium halotolerans* MDP isolate revealed that the addition of Moringa dry pod powder to the growth medium stimulated cellulase production with a yield of 0.035 ± 0.005 U/ml (3.50 ± 0.5U/gds "gram dry substrate") under the standard culture and assay conditions. Danso et al. (2022) reported a similar cellulase productivity by *Streptomyces* sp. strain MS-S2 isolated from *Microcerotermes* sp., the wood-feeding termite, using corn straw and Beech-wood xylan as inducive substrate where it yielded 0.034 ± 0.01 and 0.035 ± 0.014U/mL cellulase activity respectively. However, this recorded value is better than that recorded by the same strain when sugarcane bagasse was used as a C-source where it yielded 0.021 ± 0.005U/mL cellulase productivity. Besides, *Aspergillus flavus* has recorded the same cellulase productivity when wheat straw was used as a C-source where it reported 3.5U/gds productivity, and that value was better than that reported when rice husk and bagasse were used as a C-source (Singhal et al., 2022).

Assaying the investigated cellulase's ability to saccharify Moringa dry pod cellulose showed that, this enzyme can release 83.93 ± 15.28 mg reducing sugars from each gram of Moringa dry pod powder in 30 minutes under the standard assay conditions (8.393% w/w reducing sugars yield); that can be maintained without any chemical pretreatment steps. Montaño et al. (2017) estimated glucose recovery from saccharification of dry *Moringa oleifera* stems and leaves powder utilizing Novozyme's commercial enzymes after acid/temperature pre-treatment process. Glucose yield in their study varied from 77 to 270mg/gds in hydrolysate of water-insoluble fraction depending on the pretreatment process. Moreovver, Trivedi et al. (2015) reported a hydrolysis for *Ulva fasciata* feedstock using marine fungus cellulase. They observed that saccharification with 5U/g cellulase yields only 48.70 ±4.30, mg/gds reducing sugars.

The optimization of cellulase production by *Cladosporium halotolerans* (OP903200) was investigated as an initial step to enhance total sugar production from *Moringa oleifera* dry pod powder utilizing this fungal cellulase.

This optimization study was carried out using response surface methodology which is more accurate and efficient than the traditional time consuming 'one factor at time' method because it does not only estimate the optimal level of tested factors, but it can also estimate the mutual interaction between them (Gorbounov et al., 2022). Three important cultivation parameters were selected for this study namely, incubation temperature, inoculum size, and incubation period. Statistical analysis of the obtained model results showed the significance of the employed Box-Behnken model design. The incubation temperature and incubation period are identified as significant model terms. The decrease in incubation temperature and the increase in incubation period were estimated to enhance cellulase productivity by *Cladosporium halotolerans* (OP903200). Moreover, no mutual interaction was detected between the tested factors. One of the solutions that predicted by Design Expert's numerical optimization tool for the best cellulase productivity was selected and tested experimentally to check the accuracy of the employed model and validate it. The results showed that cellulase productivity by this isolate was enhanced to 0.115 ± 0.00U/mL when the values of the incubation temperature, inoculum size, and incubation period were shifted to 22.0, 1.0 and 13.0 respectively. This experimentally recorded value deviates from the value predicted by the employed model (0.106U/mL) by 7.96%. This deviation percent is acceptable and recommends the validation of the employed model as the predicted levels were tested with their approximated value.

The current study has estimated that the reduction in incubation temperature around 20°C has enhanced cellulase production and the increase in the temperature leads to a reduction in cellulase productivity. Similar results were reported by Trivedi et al. (2015) for *Cladosporium sphaerospermum* where the increase in incubation temperature above 25°C has obviously reduced cellulase productivity. However, Srivastava et al. (2020) indicated that the optimal temperature value for cellulase production by *Cladosporium cladosporioides* NS2 was 30°C.

Although, Trivedi et al. (2015) have reported the best cellulase productivity for *Cladosporium sphaerospermum* after incubating this isolate for 4 days after which the productivity was greatly diminished, the current study has recorded the highest cellulase productivity by *Cladosporium halotolerans* (OP903200) after its incubation for 13 days. A similar behavior was observed for

Aspergillus flavus by Singhal et al. (2022) who studied the optimization of cellulase production by this fungus employing the Response Surface Methodology-Box Behnken Design (RSM-BBD) and the best productivity was recorded after incubation for 12 days.

In this study, the employed optimization study has achieved a significant enhancement in cellulase production by the investigated fungus. It yielded a 3.3-fold increase in cellulase production by *Cladosporium halotolerans* (OP903200) compared to the productivity under the non-optimized conditions. In other studies, RSM-BBD has also been employed to enhance cellulase production by different fungal isolates. Singhal et al. (2022) obtained similar optimization results where they achieved a threefold increase in CMCase production by *Aspergillus flavus* using wheat straw. However, Saini et al. (2015) have recorded only a 1.7-fold increase in cellulase production by *Penicillium oxalicum* by employing this method. Additionally, productivity of cellulases by *Trichoderma reesei* has been enhanced by a 1.24-fold only by employing a response surface methodology optimization study (Lahiri et al., 2021).

Following this cellulase production optimization study, another optimization study has been carried out to determine the best temperature and pH values for its cellulolytic reaction. Results revealed that, the investigated enzyme shows its best activity under 50°C and pH 5. Similarly, *Cladosporium* species tested for cellulase production by Abrha & Gashe (1992) have reported the best activity at 60°C and pH 5, while cellulase produced by *Cladosporium sphaerospermum* has recorded its optimal activity under 40°C and pH 4 (Trivedi et al., 2015). On the other hand, *Rhizopus oryzae* CMCase has recorded its maximal activity at 50°C and pH 4 (Ezeilo et al., 2020). Similar results have been given for crude cellulase enzyme produced by *Penicillium oxalicum* where the best activity was maintained under 50°C and pH 5 (Saini et al., 2015).

Upon the completion of this optimization step, a further increase in cellulolytic activity has been maintained to achieve a final 4.6-fold increase in cellulase activity compared to the unoptimized conditions.

As *Cladosporium* species have been consistently isolated from low water activity habitats (reaching 0.82-0.86) such as salted and sugary foods (Musa et al., 2018), the produced cellulase's activity has been evaluated under low water activity conditions. It has been found that, the investigated cellulase retains good activity under low water activity conditions. This behavior reflects the promising applicability of this enzyme in the industrial field and in the bioethanol production process.

One of the renewable and low-cost biomass materials found in vast quantities on the Earth is lignocellulosic materials. These materials can be exploited to produce different value-added products such as bioethanol which represents a sustainable, clean, cheap, and green fuel alternative to fossil fuels (Singh et al., 2021; Devi et al., 2022). Bioethanol is commonly produced from plant materials containing high amounts of sugar, such as sugarcane, sweet potato, barley, corn, and wheat. However, using other unconsumable/non-food feedstocks in the production process is necessarily required to save these food resources. One of such unconsumable feedstocks that can be utilized in bioethanol production is the lignocellulosic materials that are produced as wastes during agriculture practices. Bioethanol produced from these materials is called the second-generation biofuel (Tusher et al., 2022). One of the least studied lignocellulosic materials that can be used for bioethanol is the Moringa pod husks. Thus, this study aims to evaluate and optimize its saccharification by new cellulases to exploit it in bioethanol production.

The efficiency of the produced *Cladosporium halotolerans* (OP903200) cellulase in saccharification of Moringa pod powder for bioethanol production without any chemical or temperature pretreatments was estimated. The saccharification process yielded an increase in the Moringa pod simple sugar content from 93.2mg/gds to 230.3mg/gds during the first 4 days, and that value was elevated to 330.6mg/gds on the 5th day. Upon inoculation of the saccharified sample on the 4th day with *Saccharomyces cerevisiae*, bioethanol production reached a concentration of 20.14g/L after 24 h and increased to 36.06g/L after 6 days of fermentation.

Ali & Jamaludin (2015) saccharified *Moringa oleifera* pod husks for bioethanol

production employing dual alkali/temperature pretreatment processes. Saccharified samples yielded only 8.40g/L bioethanol production after *Saccharomyces cerevisiae* fermentation process. Later, Ali & Kemat (2017) tested the bioethanol production from *Moringa oleifera* seed husks after their saccharification using the NaOH hydrolysis process. The highest bioethanol yield reported in this method was 29.69g/L after an optimized *Saccharomyces cerevisiae* fermentation process. On the other hand, Jin et al. (2020) estimated the saccharification of NaOH-pretreated rice straw using crude enzymes of *Aspergillus fumigatus*. This saccharification process has yielded 22.15g/L reducing sugars and 9.45g/L bioethanol production by *Saccharomyces tanninophilus* fermentation. Recently, Danso et al. (2022) tested the productivity of bioethanol from wheat straw after its saccharification by xylanase and cellulase enzymes produced by a novel *Streptomyces* sp. strain MS-S2. The maximum bioethanol yield observed in this study was 10.8g/L after *Saccharomyces cerevisiae* fermentation process.

Compared to these findings, the current study represents a new green, more efficient, and cost-effective method to produce a second-generation biofuel exploiting one of the useless and less studied agricultural waste i.e., "*Moringa oleifera* pod husks" without the need for any thermal or chemical pretreatment process.

Conclusion

The current study provides a new green, cost effective and efficient method for 2nd generation bioethanol production in a trial to solve the "fuel-food" conflict generated by the 1st generation bioethanol production process. To the best of our knowledge, this is the first study that investigates cellulase activities of *Cladosporium halotolerans*. In this study a new *Cladosporium halotolerans* isolate was isolated from decaying *M. oleifera* dry pod husks. The productivity of cellulase by this isolate was optimized by employing Response Surface Methodology Box-Behnken Design (BBD). The produced cellulase showed the best activity at pH 5 and 50°C. Upon the completion of the optimization study, a 4.6-fold increase in cellulase activity was maintained. The investigated cellulase has maintained its activity under the increased ionic strengths, and that reflects its suitability for efficient bioethanol production. Indeed, this enzyme has been efficiently used in the saccharification of Moringa dry pod powder without any pretreatment processes. Upon the inoculation of the saccharified sample with *Saccharomyces cerevisiae*, the bioethanol production reached a concentration of 36.06 g/L after 6 days. To the best of our knowledge this is the first report for the saccharification of *M. oleifera* dry pod husks by *Cladosporium halotolerans* cellulase for 2nd bioethanol production.

Competing interests The authors report no conflicts of interest regarding this work.

Authors' contributions: Design of study and conceptualization; Mohamed E. Osman and Nashwa H. Abdullah, Conducting the practical work and methodology; Nashwa H. Abdullah, Data analysis and statistics; Nashwa H. Abdullah, Resources; Mohamed E. Osman and Nashwa H. Abdullah, Writing of manuscript draft; Nashwa H. Abdullah, Editing and revision of final manuscript; Mohamed E. Osman and Nashwa H. Abdullah.

Ethics approval: Not applicable

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إنتاج الجيل الثاني من الوقود الحيوي من قشور قرون نبات المورينجا أوليفيرا بإستخدام إنزيمات تحلل السليلوز لفطر تحللي جديد : *Cladosporium halotolerans* MDP OP903200

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يمثل استنفاذ الوقود الأحفوري وتوفير الموارد الغذائية عالمياً إحدى التحديات المستقبلية الرئيسية. وعلى الرغم من أن إنتاج الجيل الأول من الإيثانول الحيوي بإستخدام المواد الغذائية السكرية قد مثل مصدرًا نظيفاً و مستدامًا لإنتاج الوقود الحيوي إلا أنه قد أدى إلى إستحداث صراع حول أولوية توفير الوقود أوتوفير الغذاء. ولذلك فقد تم الإهتمام مؤخراً بشكل كبيربكيفية إنتاج الجيل الثاني من الإيثانول الحيوي بإستخدام المواد الليجنوسليلوزية غير الصالحة للأكل. ولذلك هدفت هذه الدراسة إلى استغلال قشور قرون نبات المورينجا أوليفيرا والتى تمثل أحد المخلفات الزراعية الأقل دراسة لإنتاج الجيل الثاني من الإيثانول الحيوي باستخدام طرق نظيفة وعملية و فعالة للإنتاج من حيث التكلفة. وقد أظهرت العزلة الفطرية الجديدة *Cladosporium halotolerans* MDP OP903200 والتى تم عزلها من قرون المورينجا الجافة المتحللة تغييرات هيكلية واضحة على قرون المورينجا نتيجة نشاطها التحللي للسيليلوز. وقد تم تحسين إنتاجية هذا الفطر لإنزيم تحلل السليلوز معملياً بإستخدام الطرق الإحصائية لتصميم التجارب بإتباع نموذج منهجية سطح الاستجابة. و قد سُجل أفضل إنتاجية لهذا الإنزيم عندما تم تعديل درجة حرارة التحضين وحجم الحقنة الفطرية وفترة التحضين إلى 22 درجة مئوية و قرص فطري واحد (8 ملم) و 13 يوم تحضيني على التوالي. كما تم تعيين أفضل درجة حرارة ودرجة الحموضة لعمل هذا الإنزيم ووُجدت انها 50 درجة مئوية ودرجة حموضة 5 على التوالي. وبالإنتهاء من دراسات تحسين الإنتاجية والنشاط لهذا الإنزيم وُجد انه قد تم تحقيق زيادة فى النشاط التحللي للسليلوز بقيمة قدرها 4.6 ضعف مقارنة بالنشاط التحللي الذى تم تسجيله تحت الظروف غير المثلى. علاوة على ذلك فقد تم تسجيل نشاط جيد لهذا الإنزيم تحت ظروف النشاط المائي المنخفض للوسط والذى يعكس إمكانية تطبيقه الواعد لإنتاج الإيثانول الحيوي. وبالفعل تم تسجيل قدرة هذا الإنزيم على التسكيرالمباشر لمسحوق قرون المورينجا الجافة دون اى عمليات معالجة سابقة وتسجيل زيادة في محتوى السكرالأحادي به من 93.2 إلى 330.6 ملجم لكل جرام جاف لمسحوق القرون وأنتاج 36.06 جم/لتر من الإيثانول الحيوي عند التخمير بواسطة فطر الخميرة *Saccharomyces cerevisiae*.