Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(5): 463 – 481 (2022)
www.ejabf.journals.ekb.eg

Lignocellulosic substrate as a low-cost effective inducer for production of hydrolytic cellulases by marine halophilic *Aspergillus ochraceus*

Taghreed S. Alnusaire[1] and Aida M. Farag[2]
1. Biology Department, Collage of Science, Jouf University, Sakaka, Saudi Arabia.
2. National Institute of Oceanography and Fisheries, Egypt.

***Corresponding author:** aidafarag92@yahoo.com

ARTICLE INFO
Article History:
Received: June 28, 2022
Accepted: Sept. 13, 2022
Online: Sept. 30, 2022

Keywords:
Aspergillus,
Mangrove,
Cellulases,
Lignocellulosic substrates,
Plackett-Burman statistical design

ABSTRACT
Enzymes are one of the key materials widely recognized for their diverse applications in industry. The cellulase enzymes are used for many industrial applications such as in food, paper, biofuel, textile, and industries. The cellulolytic potential of a marine isolate, *Aspergillus ochraceus* was studied by growing it on different carbon and nitrogen sources. The impact of the introduction of lignocellulosic substrates separately into the fermentation medium on enzyme productivity was studied. Results revealed that *Aspergillus ochraceus* produced maximum activity of Fpase and CMCase in the presence of wheat bran as a sole carbon source under submerged fermentation compared to solid-state fermentation after 3 days of incubation. Statistical screening of cultural conditions for the highest enzyme production by marine halophilic *Aspergillus ochraceus* was carried out using Plackett–Burman statistical design. The design showed that the optimized media enhanced CMCase and Fpase activities by 2.87 and 2.17-fold, respectively, higher than that recorded with the basal cultural conditions.

INTRODUCTION

Cellulose is an abundantly polysaccharide that constitute most of many materials such as plant cell wall, agro-wastes, municipal wastes, and forest residues. It is a simple linear polymer consisting of β-1-4 linked glucopyranose units. Physico-chemical hydrolysis methods of lignocellulosic have remained limited for pretreatment processes such as acidic, alkaline, steam explosion, and ammonia fiber explosion **(Wyman *et al*., 2014)**. Moreover, the hydrolysis of lignocellulosic substrates is conducted by enzymatic reaction than chemical reaction due to the absence of sugar degradation in the enzymatic process.

Mangrove communities are highly ecosystem which provides large quantities of organic matter to the adjacent coastal water in the form of detritus. Hence, it is rich in energy and contains a large active microbial population both attached and living free **(Bhat, 2000)**. As cellulose is not soluble like other substance, bacterial and fungal degradation occurred exocellularly and the products of cellulose hydrolysis are available as carbon and energy

sources for other microbes that inhabit in these environments. Mangrove is composed of thick organic matter mixed with sediment, hence it is anaerobic except the sediment surface. The decomposition of cellulose such as mangrove leaves and woods are brought about by complex communities of interacting microorganisms by hydrolyzing the β-1, 4-glycosidic linkages of cellulose by enzymes such as cellulase produced by numerous microorganisms. The cellulases enzymes produced by these microorganisms have attracted much interest for the diversity of their applications.

Cellulose can be degraded by cellulases which have different specificities to hydrolyzing glycosidic bonds into simple sugars. The complete enzyme hydrolyses of cellulose requires three enzymes, endoglucanases (endo-1,4-β-glucanases, EC 3.2.1.4), exoglucanases (exo-1,4-β-glucanases, EC 3.2.1.91) and β- glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) which have high specificity for the β-1.4 glycosidic linkages in cellulose **(Lynd *et al*., 2000)**. Recently, β-D-glucoside glucohydrolase has gained an increased attention due to its numerous potential applications in the bioconversion of hemicelluloses to sugars, ethanol and pharmaceuticals, and others. Additionally, it plays a key role in improving the nutritional quality of silage and green-feed, paper and pulp industry, clarification of juices and wines, agricultural-waste treatment processes and deinking processes of waste paper **(Bhat, 2000)**. Consequently, many industries have increased their demand for finding cost-effective cellulase producing microorganisms such as bacteria **(Immanuel *et al*., 2006)** and fungi **(Siva *et al*., 2022)**. Filamentous fungi are a preferred source for commercial enzyme production due to their higher yield level compared to bacteria and yeasts **(Bakri *et al*., 2003; Kaur *et al*., 2020)**. Almost all members of genus *Aspergillus* are synthesize cellulase; thus, this genus has the potential to dominate the enzyme industry **(Siva *et al*., 2022)**.

The use of purified substrates for the production of enzymes is uneconomical route; hence, using low-cost lignocellulosic substrates for cellulases production can reduce their cost **(Ismail & Hassan, 2020)**, especially under submerged fermentation.

Different approaches such as one variable at a time (OVAT) and Plackett-Burman Design methodology (PBD) have been used for the optimization of various process parameters during the fermentation process. Most researchers used OVAT, but it requires more time and interaction of medium components with each other which haven't been studied yet. PBD is widely used to overcome these problems because it can do more experimental trials with accuracy within a short time, and each medium component has an interaction with each other **(Farag *et al*., 2015)**.

The present study aimed to evaluate cellulases production by different fungi isolated from mangrove environment, the Red Sea coast, Egypt. The most potent fungal isolate was identified. Additionally, different parameters for cellulases production were optimized.

MATERIALS AND METHODS

Sampling area

In the present work, fungi were isolated from mangrove sediment at different locations of the Red Sea coasts, Egypt (Table 1). The collected samples were kept in plastic pages and directly transferred to the laboratory.

Table 1. The study sites showing their Latitudes and Longitudes

Name of site	Latitude			Longitude		
North Safaga	26	47	34.9	33	56	12.6
Middle Safaga	26	30	20	34	0	20
North Quseir	26	12	15	34	13	15
Middle Quseir	26	8	30	34	14	30
Abou-Ghoson	25	4	6.1	34	45	0.4

Isolation of microbes from sediment samples

The samples were serially diluted and one ml of each diluted sample was plated on modified Czapek's-Dox medium (MCD) supplemented with carboxymethyl cellulose (CMC) as a sole carbon source and Congo Red (CR) dye **(Jasani *et al.*, 2016)**. The initial pH of the medium was adjusted to 6.0± 0.2 with diluted HCl or NaOH, prior to sterilization. The plates were incubated at 30°C for 3-10 days. The hydrolysis of CMC was detected by the appearance of pale yellow halo zone surrounding the fungal colonies **(Ahmad *et al.*, 2020)**. The appeared colonies were further purified by streaking on MCD medium **(Jin *et al.*, 2019)**.

Fungal identification

The fungal isolates were identified and characterized according to morphological, cultural features and microscopic examination **(CBC, 2006; Ellis, 2007)**. The complete identification of fungi was confirmed by Mycological Center, Faculty of Science, Assiut University, Egypt.

Inoculum preparation

The isolated fungi were grown on MCD medium slants for five days at 30^oC. After incubation, conidia were scraped with 5.0ml of sterile distilled water, and the spores were counted using haemocytometer. One ml (2×10^6 spores/ml) of aliquots were used to inoculate 50ml of sterilized MCD medium dispensed in 250ml Erlenmeyer flasks, and the flasks were incubated for different times at 30^oC in a rotary shaker (120rpm).

Cellulases enzymes assay

The samples were collected after incubation and centrifuged at 6000rpm for 20min. The clear supernatant was collected from centrifugation and analyzed for the filter paper assay (Fpase) and carboxymethyl cellulase (CMC ase) activity **(Ferrari *et al*., 2014)**.

Determination of filter paper assay (Fpase)

It was determined according to the method of **Mandels and Weber (1969)**. 50mg weight of Whatman filter paper strips was mixed with one ml of sodium citrate buffer (0.05 M; pH 4.8) and incubated in a shaking water bath at 50°C. One ml of enzyme preparation was added to the above mixture and incubated for 60 min at 50°C. After incubation, the liberated reducing sugars were measured by the addition of 3, 5- dinitrosalicylic acid **(Miller, 1959**) and read spectrophotometery at 540nm. A control without enzyme solution was also made. The cellulase activity was expressed in Filter Paper Unit (Fpase). One unit (U) was defined as the amount of enzyme, releasing one micromole of reducing sugar from filter paper per ml.

Determination of carboxymethyl cellulase (CMCase) activity

It was determined by adding 0.5ml of enzyme preparation to 0.5ml of 0.05 M sodium citrate buffer, pH 4.5 containing CMC (1%) as a substrate. The mixtures were incubated for 30min at 50°C in a shaking water bath **(Miller, 1959; Yan & Chai, 2021**). After incubation, the reaction was stopped by the addition of 3, 5 dinitrosalicylic acid and boiled for 10-15 min. The developed color was measured by a spectrophotometer at 540nm. The amount of reducing sugar liberated was quantified using glucose as a standard. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μM of glucose per ml under the assay conditions.

Estimation of protein

The protein content was estimated according to the method of **Lowry *et al*. (1951**). A standard curve of protein was prepared using crystalline bovine serum albumin as a standard.

Optimizing of CMCase and Fpase production

One variable at a time (OVAT) approach

It was used to screen different independent variables that would either stimulate or inhibit the production of CMCase and Fpase enzymes. This approach is based on changing one variable at a time without studying the interaction among the tested variables.

Effect of different nitrogen sources

Equivalent weight of different nitrogen sources ($(NH_4)_2SO_4$, KNO_3, $NaNO_3$, NH_4NO_3, urea, peptone, tryptone, beef extract and yeast extract) were separately added into the

production medium. The flasks without nitrogen source served as a control. After sterilization, each flask was inoculated with one ml of fungal spores (2×10^6 spore/ml). The flasks were incubated at 30°C for 3 days in an orbital shaker (120 rpm). After incubation, the culture filtrate was obtained by filtration followed by centrifugation, and the clear supernatant was analyzed.

Production of CMCase and Fpase by submerged fermentation (Smf) and solid state fermentation (SSF) using different agricultural wastes as a carbon source

Different natural agricultural wastes were used in this study including sawdust, rice bran, wheat bran, lemon peels, orange peels, oat bran and corn pop. All substrates were dried for 12h in an air dryer oven at 80°C, crushed and sieved through 0.01mm sieve size. For Smf, sterilized MCD medium was supplemented either with 1% lactose or 1.0 % commercially agricultural wastes. The medium was inoculated by 2ml of fungal spore suspension (2×10^6spore /ml) and incubated for 3 days at 30°C on a rotary shaker (120 rpm) **(Arotupin & Olaniyi, 2013)**. For SSF, fermentation was carried out in 250ml Erlenmeyer flasks containing 25g agro-waste and production medium with moisture ratio of 1:0.75 (w/v). The media were sterilized by autoclaving for 20 min at 121°C. Each flask was inoculated with 2ml of spore suspension. All flasks were incubated for 3 days at 30°C. At the end of the incubation period, each culture filtrate was obtained by filtration followed by centrifugation, and the clear supernatant was analyzed for cellulases activities and protein content.

Plackett–Burman statistical design (PBD)

The optimization process of different variables using the statistical approach for maximal CMcase and Fpase production was studied by using PBD **(Plackett & Burman, 1946)**. Eleven variables were screened in twelve trail runs. For each variable, a high (+) and low (-) levels were tested. All experiments were performed in duplicates, and the averages of CMCase and Fpase activities were the responses.

The following equation was used to estimate the main effect of each variable:

Main effect = $(\sum Mi^+ - \sum M_i^-)/N$

Where, Mi^+ and M_i^- are the observations of trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2.

The experimental results were analyzed to extract independently the main effects of these variables; the analysis of variance technique was then applied to determine which factors were statistically significant. The Student's *t*-test was employed to check the statistical significance of the regression coefficients of the variables. Significant variables can be measured by probability *P*-value. If *P*-value < 0.05 for the particular variable, then that variable is said to be statistically significant. Minitab 15 Statistical Software (Minitab,

Inc., State College, PA) was used for the statistical analysis of the experimental data.

Statistical analysis

All measurements were carried out in triplicate. The results were presented as mean values ± SD, standard deviations.

RESULTS

Qualitative screening for extracellular cellulases production by the isolated fungi

A total of twenty five fungal strains were isolated from different mangrove sediment samples from the Red Sea coasts, Egypt. The isolated fungi were identified according to physiological and morphological characteristics to four genera, *Aspergillus, Penicillium, Rhizopus* and *Fusarium.* The isolated fungal species were screened for their capabilities to produce cellulases using MCD agar medium. Results indicated that all isolates were positive producers with different efficiencies. The clear zone diameter can be used as a quantitative comparison of cellulolytic activity of the different isolates as the clear zone diameter range was 8–22mm (Table 2). The efficient isolate which showed the highest zone diameter was selected for further experiments.

Effect of incubation period on enzymes production

The results (Figs. 1A, B) show that *A. ochraceus* was the most cellulases producer species. The CMCase and Fpase activities of *A. ochraceus* increased by increasing the incubation time and reached the highest values (24.127± 0.965 and 11.29± 0.805 U/mg protein, respectively) after 3 days of incubation. On the other hand, both *A. flavus* and *A. terreus* produced the highest CMCase (19.576±0.444 and 16.642±0.765 U/mg protein, respectively) and Fpase (8.87±0.505 and 10.34± 0.552 U/mg protein, respectively) after 5 days of incubation. Consequently, the fungal strain *A. ochraceus* was chosen as the most potent cellulases producer.

Optimization of CMCase and Fpase production

Effect of different nitrogen source

The results (Fig. 2) clearly revealed that all organic nitrogen sources enhanced both CMCase and Fpase enzymes production compared to the inorganic ones. The highest CMCase and Fpase activities (44.934±0.774 and 40.690±0.523 U/mg protein, respectively) by *A.ochraceus* were detected in cultures supplemented with peptone followed by yeast extract.

Table 2. Clear halo zone diameter (mm) resulted from hydrolysis of CMC by marine isolates

Isolate code	Isolate (suspect)	Cultural characteristics	Microscopic examination	Halo zone diameter (mm)
AS1	*Aspergillus terreus*	Brownish and dark on its ages on culture.	Conidial heads are compact, densely columnar and biseriate. Conidia are small, globose-shaped and their color varied from hyaline to light yellow.	16
AS2	*Aspergillus ochraceus*	yellow mold and grow rapidly on agar	Conidiophores appear as a powdery mass.	22
AS3	*Aspergillus niger*	Black mycelia	Simple, upright conidiophores terminating in ovoid swelling	10
AS4	*Aspergillus flavus*	Greenish yellow mycelial growth	An upright conidiophores that terminates in a davate swelling bearing phialides at the apex or radiating from the entire surface; conidia are 1-celled and globose	18
P1	*Pencillium* sp.	Blue mold growth	Septate mycelia bearing single conidiophores which are branched near the apex ending in phialides that carry the conidia	10
R1	*Rhizopus* sp.	Rapidly growing white colored fungus, swarms over entire plate	Non-septate mycelia, sporangiophores bearing sporangium with a columella and root- like hyphae (rhioziod) penetrating the medium	9
F1	*Fusarium* sp.	White mycelia with areas of whitish yellow	Fast growing rate with aerial mycelium. They appeared to be sickle-shaped.	8

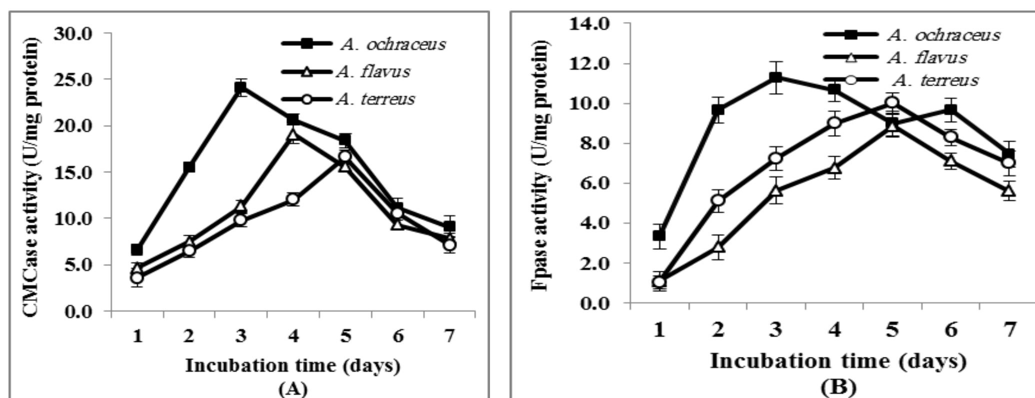


Fig. 1. Effect of fermentation time course on the production of CMCase (A) and Fpase (B) by different fungal isolates

Data are represented as mean ±SD across technical replicates (n = 3).

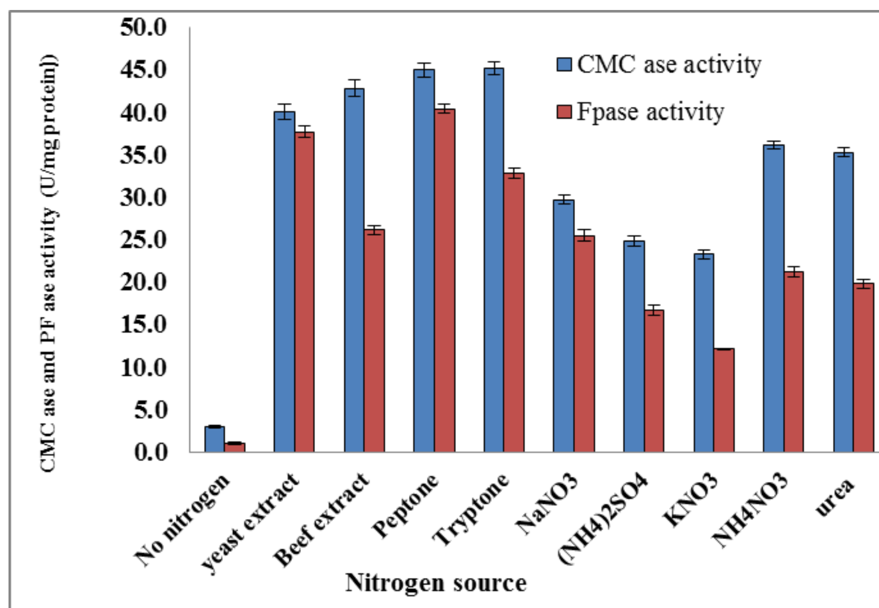


Fig. 2. Effects of different nitrogen sources on CMCase and Fpase production by *A.ochraceus* cells

Data are represented as mean ±SD across technical replicates (n = 3).

Effect of different lignocellulose substrates on CMCase and Fpase production

The activity of cellulases enzymes varied significantly on different lignocellulose substrates under SmF, and SSF (Figs. 3A, B). Among all substrates examined, wheat bran was the best one producing the highest CMCase and Fpase enzymes activates in both SmF(65.139±1.01 and 59.12±1.03 U/mg protein, respectively) and SSF (52.55±0.909 and 50.37±0.858 U/mg protein, respectively). On the other hand, orange peels produced the lowest enzymes activities for CMCase and Fpase in both SmF and SSF. This variation may be due to nutrient availability and the chemical nature of each substrate.

Optimization of culture conditions using PBD

In the current study, PBD was used to screen the variables significantly affecting CMCase and Fpase production by marine *halophilic A. ochraceus*. The examined variables and their levels are presented in Table (3). The enzymes' activities at the variables tested are shown in Table (4). There was a variation in CMCase activity ranging from 29.45 to 112.76 U/ mg protein and Fpase activity from 20.45 to 153.23 U/mg protein, which reflects the importance of studying the medium composition and cultural conditions for obtaining the highest productivity. In addition, the main effects of the examined variables on CMCase and Fpase production were calculated and plotted in Fig.

(4), which displays the magnitude of each estimate and shows the ranking of the variable estimates.

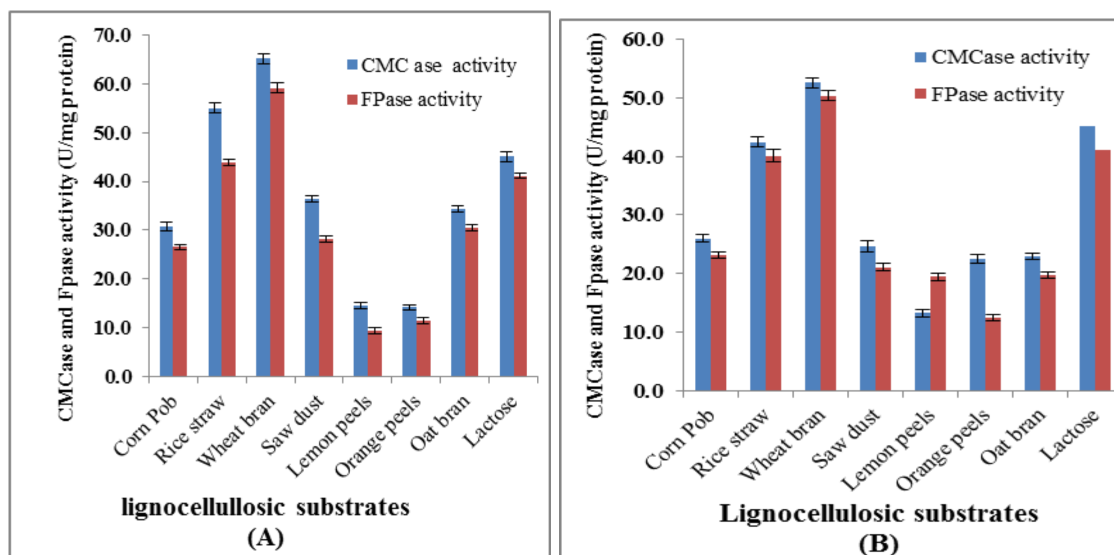


Fig. 3. Effects of different lignocellulosic substrates on CMCase and Fpase production by *A.ochraceus* culture under SmF (A) and SSF (B).
Data are represented as mean ±SD across technical replicates (n = 3).

All data on the experiments were statistically analyzed using the analysis of variance. Significant findings were found based on calculated *t-test* (Table 5), the high Fischer's test F-value, and low probability *P*-value ($p < 0.05$). It was found that wheat bran, KCl, $MgSO_4$, temperature, volume of medium, size of inoculum, and shaking speed had a positive effect on CMCase production within the test ranges; while, peptone, K_2HPO_4, $FeSO_4$ and pH had a negative effect on CMCase production. Moreover, the regression coefficients for the eleven examined variables for Fpase production showed that all had a positive effect on Fpase production, except peptone, K_2HPO_4, $FeSO_4$ and volume of medium which showed a negative effect (**Figure 4**). The goodness of fit of the model was checked by determination of the correlation coefficient (R^2). R^2 value was calculated to be 0.9998 for CMCase production, indicated that about 99.98% of the total variability in the response could be explained by this model and only 0.02% of the total variation were not explained. Also, the model coefficient of determination (R^2) of Fpase production has a value of 0.9995, which points out that 99.95% of the response variability could be explained by the model and only 0.05% of the total variation was not explained.

Table 3. Experimental variables at different levels used for CMCase and Fpase production, using Plackett–Burman design

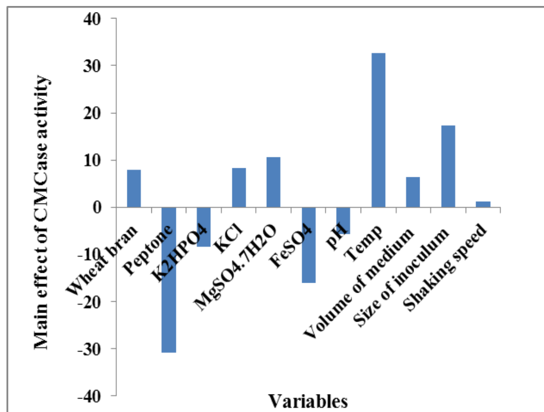
Variables (g/l)	Symbol code	Experimental values Lower (-1)	Basal (0)	Higher (1)
Peptone	P	2.5	5	7.5
Wheat bran	W	5	10	15
K_2HPO_4	KH	0.5	1.0	1.5
KCl	K	0.25	0.5	0.75
$MgSO_4.7H_2O$	Mg	0.25	0.5	0.75
$FeSO_4$	Fe	0.01	0.02	0.03
pH	pH	4.0	6.0	8.0
Temperature (^oC)	T	25	30	35
Volume of medium (ml)	V	25	50	75
Size of inoculum (ml/50 ml)	IS	1.0	2.0	3.0
Shaking speed(rpm)	Sh	80	120	160

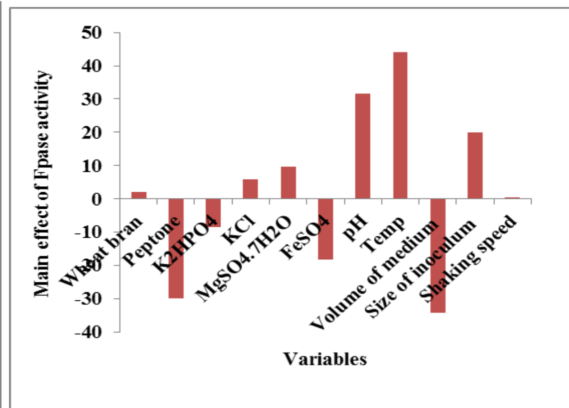
Table (4). Plackett–Burman design matrix for eleven variables with coded levels

Trail	p	W	KH	K	Mg	Fe	pH	T	V	IS	Sh	Actual CMCase (U/mg protein)	Predicted CMCase (U/mg protein)	Actual Fpase (U/mg protein)	Predicted Fpase (U/mg protein)
1	1	1	1	-1	1	1	-1	1	-1	-1	-1	**112.76**	**112.720**	**153.23**	152.996
2	1	-1	1	-1	-1	-1	1	1	1	-1	1	**53.83**	**53.792**	**50.11**	49.876
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	95.20	95.162	130.34	130.106
4	-1	1	-1	-1	-1	1	1	1	-1	1	1	99.71	99.670	58.55	58.316
5	1	1	-1	1	-1	-1	-1	1	1	1	-1	92.86	92.822	40.23	39.996
6	1	-1	1	1	-1	1	-1	-1	-1	1	1	49.33	49.285	70.57	70.336
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	101.11	101.069	58.34	58.106
8	-1	-1	-1	1	1	1	-1	1	1	-1	1	29.45	29.410	20.45	20.216
9	1	1	-1	1	1	-1	1	-1	-1	-1	1	53.11	53.069	54.22	53.986
10	-1	-1	1	1	1	-1	1	1	-1	1	-1	57.75	57.706	50.66	50.426
11	1	-1	-1	-1	1	1	1	-1	1	1	-1	39.72	39.677	29.555	29.321
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	50.18	50.138	29.555	29.321
13	0	0	0	0	0	0	0	0	0	0	0	69.05	69.543	59.11	61.917

Table 5. Statistical analysis of the Plackett-Burman experiment for CMCase and Fpase activities

Intercept	CMCase activity					Fpase activity				
	Coefficients	Standard Error	t- Stat	P-value	Confidence level (%)	Coefficients	Standard Error	t- Stat	P-value	Confidence level (%)
	69.543	0.143	484.981	0.001		61.917	0.810	76.413	0.008	
P	-15.393	0.149	-103.136	0.006	99	-14.908	0.843	-17.677	0.036	96
w	3.935	0.149	26.364	0.024	98	1.075	0.843	1.275	0.424	58
KH	-4.218	0.149	-28.262	0.023	98	-4.185	0.843	-4.962	0.127	87
K	4.172	0.149	27.952	0.023	98	2.965	0.843	3.516	0.176	82
Mg	5.351	0.149	35.852	0.018	98	4.855	0.843	5.757	0.109	89
Fe	-8.002	0.149	-53.614	0.012	99	-9.093	0.843	-10.781	0.059	94
pH	-2.868	0.149	-19.217	0.033	97	15.761	0.843	18.688	0.034	97
T	16.370	0.149	109.685	0.006	99	21.981	0.843	26.063	0.024	98
V	3.198	0.149	21.425	0.030	97	-17.076	0.843	-20.247	0.031	97
IS	8.700	0.149	58.293	0.011	99	9.908	0.843	11.748	0.054	95
Sh	0.593	0.149	3.972	0.157	84	0.207	0.843	0.246	0.846	15
Multiple R	0.9999					0.9998				
R Square	0.99998					0.9995				
Adjusted R Square	0.9996					0.9942				





(A) **(B)**

Figure 4. The main effect of different variables on CMCase (A) and Fpase (B) production by *A.ochraceus* , based on the results of Plackett–Burman design.

Figure 5 shows the ranking of variable estimates in a Pareto chart. It displays the magnitude of each variable and is a convenient way to view the results of a Plackett–Burman design (**Jain *et al*., 2010**). The length of each bar on a standardized Pareto chart is proportional to the absolute value of its associated regression coefficient or estimated effect.

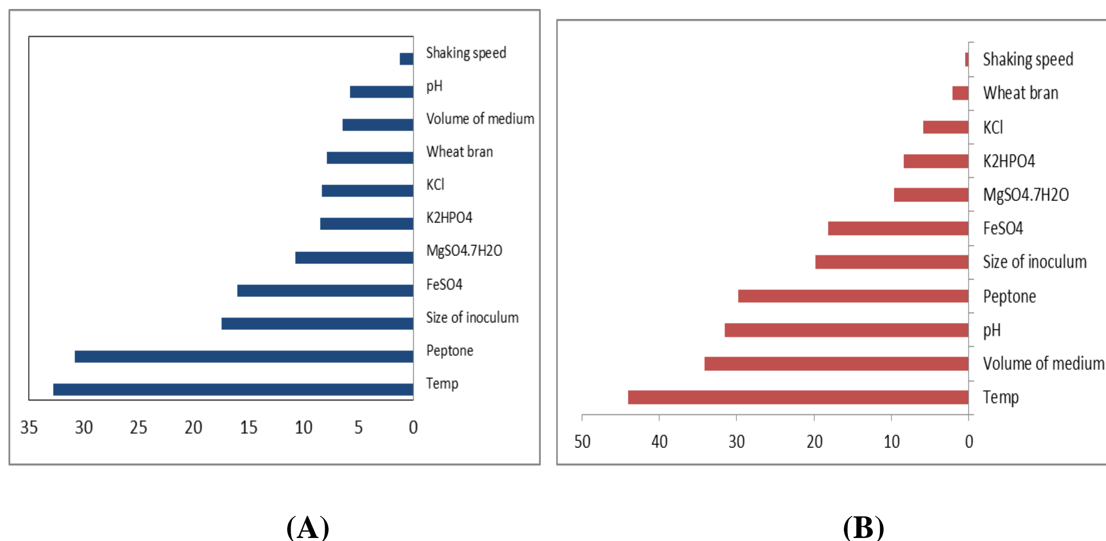


Figure 5. Pareto chart for Plackett-Burman parameters estimates (A) for CMCase and (B) for Fpase activity.

Verification of the model

In order to verify the obtained results and to evaluate the accuracy of statistical design, a verification experiment was carried out. The predicted near optimum medium and far from optimum levels of the independent variables were examined and compared to the basal condition. CMCase activity is predicted with the following medium composition (g/l): Wheat bran, 15.0; peptone, 2.5; K_2HPO_4, 0.5; $MgSO_4$, 0.75; KCl, 0.75; $FeSO_4$, 0.01; inoculum size, 3%; pH, 4.0, and volume of medium, 75 ml after 3 days of incubation at 35 °C and shaking speed 160 rpm. Also, an optimum response for Fpase activity is predicted with the following medium composition (g/l): Wheat bran, 15.0; peptone, 2.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.75; KCl, 0.75; $FeSO_4$, 0.01; inoculum size, 3%; volume of medium, 25 ml, pH 8.0, incubated at 35° C for 3 days under shaking conditions (160 rpm). Under the optimized condition, the CMCase and Fpase activities were 116.35 and 158.11U/mg protein, respectively. These results indicate that the optimized conditions accelerated the CMCase and Fpase production about 1.67 and 2.67-fold higher than that recorded with the basal condition.

DISCUSSION

Production of enzymes is the first crucial and important step in enzyme technology which needs much attention for the economic feasibility of the process (**Singh *et al*., 2021**). Fungi, specially, ascomycetes can produce diverse set of oxidative and hydrolytic enzymes such as cellulases and hemicellulases. Cellulases have been produced from filamentous fungi, specially, *Aspergillus* sp., *Trichoderma* sp., and *Penicillium* sp., that are of great interest due to their great capacity to produce plant cell wall-degrading enzymes used to convert lignocellulosic substrates to fermentable sugars (**Passos *et al*., 2018; Li *et al*., 2020**).

Twenty five fungal strains were isolated from different sediments samples as producers of cellulases enzymes according to the production of halo zone on the MCD agar medium. The fungal isolates were identified morphologically and microscopic according the manual of fungal Atlases (**CBS, 2006; Ellis *et al*., 2007**) to four genera *Aspergillus*, *Rhizopus, Pencillium* and *Fusarium*. The clear halo zone produced by isolate AS1 (*Aspergillus ochraceus*) is the largest (22 mm) among all fungal isolates, but it is not necessarily selected to be used as a potent isolate for cellulase production. The plate screening method is only qualitative not quantitative and the correlation between enzyme activity and colony-to-clear-zone ratio is considered to be weak (**Maki *et al*., 2009**). Also, the fungal isolate AS1 (*A. ochraceus*) showed the highest CMCase and Fpase activities among all others fungal strains. These differences may be due to the source of isolation and variation in genetic makeup of each isolate (**Arotupin and Olaniyi, 2013**). The short time of incubation for enzyme production may offer the potential for low-cost production **(Sharma *et al*., 2013**). An increase in the incubation time may reduced the enzyme production due to the depletion of micro and macronutrients in the fermentation medium which stressed the fungal physiology and lead to inactivation of secreting the enzymes (**Devanathan *et al*., 2007**).

The potential cellulolytic enzyme production is continuing in the interest of successful bioconversion of lignocellulosic biomass. Therefore, the present work highlights the isolation of locally cellulose degrading marine fungi from mangrove environment, Red Sea Coast, Egypt. Different microorganisms can degrade lignocellulosic biomass in mangrove environments around the world included bacteria, fungi, yeast, and actinomycetes (**Devanathan *et al*., 2007 and Gilna and Khaleel, 2011;**). **El-Morsy (2000)** isolated several cellulose degrading fungi named as *Aspergillus niger*, *Penicillium chrysogenum* and *Chaetomium hamadae* from mangrove roots of *Avicennia marina*, Red Sea Coast, Egypt.

Cellulases enzymes production have been affected by many factors such as carbon, nitrogen, pH, and others. The nitrogen sources play an important role in the growth of the organism and the production of different enzymes as organisms use it as secondary energy sources. The nature of nitrogen added to the production medium whether in the

form of organic or inorganic source might stimulate or down modulate the production of enzymes (**Prabakaran *et al.,* 2015**). Also, organic nitrogen have high amino acid content and good source of carbohydrates and vitamins which can stimulate the enzymes production (**Sharma and Thakur, 2018**). The current results showed that organic nitrogen source peptone was very effective in enhancing both CMCase and Fpase production. Similarly many reports used different organic nitrogen sources for enhancement cellulases yield (**Coban and Biyik, 2011).**

There are two types of fermentation techniques: solid-state fermentation (SSF) and submerged fermentation (SmF) that were generally used and studied in the production of cellulases enzymes (**Hagaggi, 2018**). Production of many microbial enzymes in SmF has been preferred by the industry due to better control of parameters in the whole process as temperature, agitation, aeration, foam, and pH, depending on the specific reactor type (**Aishwarya *et al*., 2011**). These parameters are very important for the highest product yield. Most filamentous fungi can produce spherical pellets when grown in a submerged culture, and this morphological difference compared to SSF provides a possible explanation for the observed enzyme production (**Znidarsic, 2001**). One of the major benefits of SmF is the straightforward of the product purification process. SmF is widely used for the extraction of secondary metabolites that are secreted into the growth medium (**Naher *et al*., 2021). Bakri *et al*. (2010)** made a comparison between enzyme production in SSF and SmF and found that the maximum enzyme production was found for the enzyme extracts from SmF compared to SSF. Many studies reported that wheat bran can be induced higher cellulase activity of many fungi when used as a substrate in both SmF and SSF **(Devanathan *et al*., 2007; Anita *et al*., 2009).** Nevertheless, many lignocellulose substrates were used for cellulase production by different fungal species such as rice straw (**Aggarwal *et al*., 2017**) and rice bran (**Navaneethapandian *et* al**., **2021**).

The nutritional and environmental conditions have a great influence on production of microbial enzymes. The medium composition and culture conditions play a significant role in enzymes production. Experimental designs such as Plackett-Burman design was used intensively for screening nutritional and cultural conditions that affecting the production of many enzymes and other metabolites (**Ashour *et al*., 2016**; **Farag *et al*., 2018**). Out of tested variables, peptone, K_2HPO_4, and $FeSO_4$ were negatively influenced both CMCase and Fpase production significantly. It can be explained by the fact that these minerals may present in wheat bran substrate and support the fungal growth as well as enhance enzymes production. The data revealed that the enzymes production by *A. ochraceus* culture under static conditions were lower than shaked conditions because agitation is a very important factor in the fermentation process that increases the amount of dissolved oxygen in the medium. Also, in static cultures, biomass was formed at the surface, therefore contact of nutrients and oxygen was low (**Subasioglu and Cansunar,**

2010). The shaking speeds for maximum cellulases production by many fungi were varied in the range of 100-200 rpm (**Li *et al*., 2020**).

PBD addresses the evaluation of variables effects on cellulases production in terms of calculating the p-value and coefficients of each tested component. The smaller the P-values, the bigger the significance of the corresponding coefficient (**Heck *et al*., 2005**). Also, confidence levels greater than 90% (**Abdel-Fattah and Olama, 2002**) or 95% reveals a significant effect on the response (**Niladevi *et al*., 2009**). The Pareto chart displays the magnitude of each variable determine (independent on its contribution, either positive or negative) and is a convenient way to view the results of a Plackett-Burman design (**Farag *et al*., 2018**). These optimum conditions have raised the average production of CMCase and Fpase enzymes by *A.ochraceus* culture by 1.67 and 2.67- fold increase, respectively, when compared to the conditions applied before optimization.

PBD was used for improvement the production of microbial enzymes by different microorganisms (**Farag *et al*., 2015; Othman *et al*., 2015 and Ashour *et al*., 2016**; **Ahmad *et al*., 2020**).

CONCLUSION

The current study searching for local marine fungal isolates that produce cellulases enzymes. It suggested that marine halophilic fungal isolate *Aspergillus ochraceus* isolated from the mangrove sediments habitat had the potentiality for CMCase and Fpase production. Also, *A.ochraceus* produced high levels of CMCase and Fpase enzymes by using wheat bran as the best carbon source under submerged fermentation. Optimization of the fermentation conditions and the medium components was carried out using one variable at a time experiment and Plackett–Burman design, indicating a 1.67 and 2.67-fold increase in CMCase and Fpase activities, respectively. Therefor, the present work indicated the possible use of marine halophilic *A.ochraceus* in the production and enhancement of CMCase and Fpase activities using wheat bran . It may be useful for the industrial production of the cellulases enzymes as an easier and cheaper method.

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