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Fermentation of Multi-Targeted Products: A statisticalApproach Discussed the Production of Cell Wall Hydrolytic Enzymes from *Streptomyces rochei* for Pathogenic Fungi biocontrol

E. W. Elgammal*, E. F. Ahmed, M. M. Abdel Aziz

Chemistry of Natural and Microbial Products Department, National Research Centre, (ID: 60014618), 33-El Bohouth st., Dokki, Giza, Egypt, P.O.12622

Abstract

The production of synergistically multi-acting cell wall hydrolyzing enzymes such as chitinase, glucanase, protease and whole cell-wall lytic enzymes "WCL" was highly observed in the culture filtrate of a strain molecularly characterized as *Streptomyces rochei* MZ227230. Enzymes productivity was monitored in different media and superiority of the modified lytic enzymes production medium(M4) was clearly noticed. Then the simultaneous optimization production of these enzymes through application of statistical modelling was conducted. Box-Behnken design (BBD) using the key components of M4 (*C. albicans*cell walls, baker yeast, chitin and peptone) followed by independent analysis for productivity of each enzyme helped to conduct four significant models describing four media that were specifically optimized to produce the utmost yields of chitinase (14.97), glucanase (27.89), protease (137.59) and WCL (65.28 U/ml). Pearson's correlation analysis indicated the impossibility to potentiate the tested enzymes to the utmost yields simultaneously. However, the coproduction medium potentiated more than 80% of the maximal yields for all enzymes. Furthermore, the crude filtrate showed potent fungicidal effect against *Fusarium graminearum, Mucor racemosus, Fusarium solani,* and *Candida albicans* which should be attributed to its hydrolytic activity as indicated by the dose and time-dependent release of DNA, RNA and protein constituents of *C. albicans*.

Keywords: Multi-cell wall lytic enzymes, *Streptomyces rochei*, biological control, statistical modelling

1. Introduction

Lytic enzymes or cell wall degrading enzymes are consisting mainly of more than one enzyme such as glucanase, chitinase and protease which are able to hydrolyze glucans, chitin and proteins as the main skeletal components in fungal cell wall by acting synergistically to hydrolyze the pathogen cell wall [1,2]. The synergistic effect starts with protease which acts on cell wall protein to open its structure and expose the inner layer of glucan and chitin to be attacked by glucanase and chitinase forming a big hole in the cell wall and plasma membrane [3]. Therefore, this system of enzymes can be categorized as biological control agents [4] and considered as eco-friendly products [5]. They are produced from different microorganisms as antagonistic key factors against several pathogens [2,6].

Lytic enzymes have broad industrial applications such as extraction of proteins, enzymes and carbohydrates, protoplast formation, control of pathogenic fungi and degradation of yeast cell mass [7]. So, lytic enzymes as safe and effective natural products have the priority to apply in the field of biological control of plant diseases instead of the chemical fungicides which can accumulate in the animal and human food causing high toxicity [5]. Furthermore, they can be applied as antimicrobial agents against the multidrug resistant microbes. Since the antimicrobial resistance patterns are partly dependent on their mechanism of action, so the mode of action must be investigate [8]. Targeting more than one type of lytic enzymes to be simultaneously optimized is highly advantageous; it could strongly potentiate the antifungal activity of studied microbial culture and will afford fermentation of multiple financial returns. However, simultaneous optimization of many products in the same fermentation (fermentation of multi-targeted products) has some practical constrictions; each of co-products has its own favorable requirements that may not support or even contradict the production of others, and so simultaneous optimization may achieve an increase in

*Corresponding author e-mail: emanelgammal50@yahoo.com.
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the production of each product yet to level that is more or less close to the maximum yield obtained at fermentation of mono-targeted product.

Many investigators were engaged with optimization studies on co-production of several products [9,10,11,12,13] yet they did not compare the yield obtained under simultaneous optimization with the yield obtained under single-product optimization. The question of "*to how extent could the yield of a product in a fermentation of multi-targeted products be close to the yield obtained in fermentation of mono-targeted product*" is well valued for assessing the economic feasibility of fermentation of multi-targeted products and of course will encourage more future studies aiming at getting more proficiency in such fermentation. As such, the current study targeted the simultaneous optimization in production of some lytic enzymes through the effective tool of statistical modelling and evaluated the efficiency of optimization by comparing the yields obtained with those attained in mono-targeted fermentation. The study has also extended to apply the culture containing maximum coexistence of lytic enzymes in *in-vitro* control of some pathogenic fungi and followed the release of vital fungal cell constituents.

2. Experimental

2.1. Microorganisms, growth conditions and inoculum preparation

Some soil *Streptomyces* isolates were screened for lytic enzymes production using modified TLE medium[14]. The modified medium is containing % (K_2HPO_4: 0.3, KH_2PO_4; 0.2, $MgSO_4$. $7H_2O$: 0.03, Peptone: 0.05, Glucose: 0.02, Chitin: 0.1, Baker yeast: 0.5, $NaNO_3$: 0.1 and cell wall of *C. albicans*: 0.3). The enzyme system production was determined after 4 days of incubation at 30°C and 200 rpm to detect the most producing isolate. The most potent organism [isolate 20S] was grown in ISP2 agar slants for 5 days at 30ºC and kept at 4°C. Then, two mL of the sporesuspension (10^6 CFU/ml) were inoculated into 50 ml of the fermentation medium. The cultures were incubated in a shaking incubator at 200 rpm for 4 days at 30ºC. After that, the enzymatic activity was determined in cell free supernatant.

2.2. Identification of the most potent streptomyces isolate using 16S rRNA method

Molecular identification of isolate 20S using 16S rRNA method was done according to Sigma Scientific Services Company method as follows:

DNA extraction was made by Quick-DNA Bacterial/Fungal Micro-prep Kit (Zymo research #D6007). Then PCR cleanup to the PCR product was made by using the GeneJET PCR Purification Kit (Thermo K0701) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Finally, the sequencing was made to the PCR product on GATC Company by an ABI 3730xl DNA sequencer using 27F forward primer (5'AGAGTTTGATCMTGGCTCAG3') and the universal bacterial reverse primers 1492R was 5' GYTACCTTGTTACGATT3'.

2.3. Assay of lytic enzymes

2.3.1 Preparation of cell wall substrate

Five liters of heat killed *C. albicans* were sonicated and centrifuged then the residual cell walls were washed then lyophilized and stored according to Valois et al. [15].

2.3.2 Turbidimetric method

Whole cell-wall lytic activity (WCL) was detected by measuring the lysis extent of heat-killed *C. albicans* suspension at 660 nm according to Tominaga and Tsujisaka[16] with some modifications in volumes of the reaction mixture. One unit of lytic activity is defined as a change in absorbance of 0.001 at 660 nm of a heat-killed suspension of *C. albicans* per hour.

2.4. Glucanase assay

The enzyme activity was detected spectrophotometrically by 3,5-dinitrosalicylic acid reagent (DNS) method [17]. The reaction mixture containing 0.5 ml enzyme and 50 µl laminarin (2% dissolved in 50 mM sodium acetate buffer, pH.5.0) was incubated for 30 min at 37°C followed by addition of 3 ml DNS reagent (10 g 3,4-dinitrosalicyclic acid. 403 g potassium sodium tartrate tetrahydrate and 16 g NaOH) to stop the reaction. After boiling in water bath for 10 min, the reducing sugar was measured at 540 nm. One unit of enzyme (U) was defined as the amount of protein necessary to produce one microgram of reducing sugar per min.

2.5. Chitinase assay

N-acetylhexosaminidase activity was detected according to Coudron et al. [18]. A sample of 50 µl of enzyme filtrate was added to 50 µl of p-Nitro phenyl-β-N-acetylglucosaminide (1 mg/ml in 0.05 M acetate buffer, pH 5) and mixed then incubated in shaking water bath for 1 hour at 30°C. The reaction was stopped by addition of 2.5 ml of 0.125 M sodium borate buffer (pH 10). The amount of the released p-nitro phenol was measured spectroscopically at 410 nm. One unit of the enzyme activity was defined as the amount of enzyme that releases 1 µmol of p-nitro phenol per min under the specified assay conditions.

2.6. Protease assay

Protease activity was determined in the culture filtrate using casein as a substrate, by the modified procedure of Tsuchida*et al.* [19] method. Equal volumes (0.5 ml) of enzyme solution and substrate solution (2% w/v casein in 50 mM Sodium phosphate buffer pH 7) were mixed and incubated at 37°C for 30 minutes. To stop the reaction; 1ml of 5% (w/v) trichloroacetic acid was added then incubated in ice for 15 minutes. The insoluble proteins were precipitated

by centrifugation and the acid soluble supernatant was neutralized by adding 5 ml of the alkaline Na_2CO_3 solution (0.5 M). Finally, 0.5 ml of two fold diluted FolinCiocalteau reagent was added then the mixture was incubated at room temperature for 30 minutes. The absorbance was measured at 750 nm. One protease unit is defined as the amount of enzyme that releases 1mg tyrosine per ml per minute under the above assay conditions.

2.7. Optimization of fermentation conditions

2.7.1. Effect of different media

Modified media (g/l)
M1: Baker yeast; 5, NaH_2PO_4: 1 [20].
M2: Reynold's medium: K_2HPO_4; 0.7, KH_2PO_4; 0.3, $MgSO_4$; 0.5, $FeSO_4$; 0.01, $ZnSO_4$; 0.001, cell wall of *C. albicans;* 5 [21].
M3: Starch nitrate: Starch; 20, KNO3; 2, KH_2PO_4; 1, $MgSO_4$; 0.5, NaCl; 0.5, CaCO3; 3, FeSo4; 0.01; cell wall of *C. albicans;* 2 [22].
M4: Modified TLE medium: As mentioned in section 2.1 [14].

2.7.2. Statistical design and modelling

Box-Behnken design, analysis of variance (ANOVA) of data, regression analysis to get coefficients in the most fit equations and predictions of the optimum levels of variables were achieved using the "Design Expert" software (Version 7.0.0, Stat-Ease Inc., Minneapolis, USA) statistical package. Pearson's correlation coefficient (r) was determined by Microsoft Office Excel 2007.

2.8. In vitro antifungal effect

2.8.1 Test microorganisms and inocula preparation

Some local plant pathogenic fungi such as*Fusarium graminearum, Alternaria betroselini, Mucor racemosus, Fusarium solani,* and *Candida albicans* ATCC10231 werekindly provided from Chemistry of Natural and Microbial Products Department, Pharmaceutical Division, National Research Centre. The strains were grown and kept on Potato Dextrose Agar (PDA) slants at 4°C. To prepare inoculum, a loopful of 2-5 days old cultures was transferred into sterile saline solution to provide a final concentration of approximately 10^6 CFU/ml adjusted according to the turbidity of 0.5 McFarland scale tube at 600 nm.

2.8.2 Antifungal assay

The culture filtrate of the most potent isolate obtained from the previous step was tested for its antifungal activity by using agar well diffusion method [23]. After preparing of PDA medium and sterilization, the medium was cooled to 47°C and then poured in sterilized Petri dishes (9 cm) containing 100 µl of different inocula. After solidification, 10 mm wells were made and loaded with 100 µl of the crude culture filtrate which was sterilized by passing through bacterial filter membrane. Then the inoculated plates were incubated for 48 h at 30°C. The inhibition zone diameter expressed in millimeters (mm) was detected by measuring against 0.5 mg/ml ketoconazole obtained from bioMerieux (Marcy l'Etoile, France)as standard antifungal.

2.9. Mechanism of action of crude multi-lytic enzymes depending on cell membrane integrity

Cell membrane integrity of *C. albicans* was detected by quantification of cellular constituents released from the cell to outside medium. Different concentrations of sterilized crude lytic enzymes (100%, 75%, 50% and 25%) which contain 1 mg protein/ml were prepared and diluted in phosphate buffer saline. Then the inoculum (10^5 CFU/ml) was suspended in 5 ml saline and then 100 µl of it were added to the tested concentrations after that the samples were incubated at 30°C for 6 h. Samples were taken after 3 and 6 h and centrifuged for 5 min then the filtrate was collected and measured using NanoDrop (Thermoscientific British UK) to detect the concentration of DNA, RNA, and protein released [24].

3. Results and discussion

3.1. Screening for production of hydrolytic enzymes

During a screening course on the production of hydrolytic enzymes system by some soil *Streptomycetes*, one of the isolates (assigned as 20S) showed remarkable ability to produce whole cell wall degrading enzymes system using the yeast *C. albicans* cell walls with production activity reached 15.0 U/ml. Basing on this result, isolate 20S was found to able to show wide inhibition zone around its colonies in baker-yeast seeded medium which reflected its intense antifungal activitywhich was dependent on its ability to secret lytic enzymes (Fig. 1).

Fig. 1. Inhibition zone around the isolate 20S

3.2. Molecular identification by 16S rRNA

The isolate was molecularly characterized and its 16S rRNA gene sequence (accession number: MZ227230 in NCBI gene bank) showed close similarity to *Streptomyces rochei* (about 99%) as depicted in phylogenetic tree which conducted by MEGA6 software using maximum likelihood method (Fig. 2).

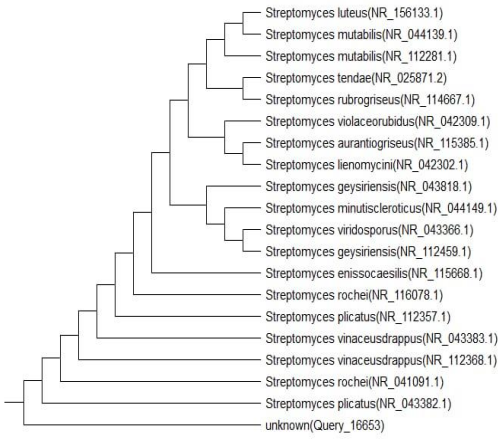

Fig. 2. Phylogenetic tree of *Streptomyces rochei strain* MZ227230

3.3. Production of hydrolytic enzymes in different fermentation media

Enzyme production is highly affected by composition of fermentation medium. As such, productivity of hydrolytic enzymes was monitored in four different media. From Table 1, it was observed that the activity values of glucanase, protease and chitinase were varied considerably depending on the composition of the applied medium and substrate added to the medium [25]. The medium (M4) supplemented with chitin, baker yeast (glucan substrate) and *C. albicans*cell wall was the best ones for biosynthesis of the three enzymes due to the presence of the inducer as main sources of carbon [26]. Also, the results revealed that activity of protease was higher than glucanase and chitinase in all tested media, this may be due to the presence of yeast biomass or baker yeast which can induce good expressions for protease [27]. As the dry matter of yeast cell wall contains about 28% of glucan and 11% of chitin [28] in addition to 40% protein [29], it was noticed that all tested media produced the enzymes protease, glucanase and chitinase in an amounts reflected their the abundance of their substrates in dry matter of yeast cell wall.

Furthermore, in this study, the production of glucanase was superior to that produced by Piegza [26} while, chitinase and protease production was lower than that obtained by Shakeri et al. [27].

3.4. Statistical optimization for maximizing the production of each enzyme

Superiority of medium M4 over other tested media was assumed to be attributed to its contents of the four main components of chitin, baker's yeast, *Candida* cell walls and peptone. The four components play crucial roles as inducers for production of the targeted enzymes. As such, they were undertaken in Box-Behnken design (BBD) to be properly formulated for attaining the highest production of the three enzymes in addition to WCL. The four components were coded and applied at three levels as shown in Table 2. The Box-Behnken design of 27 runs with three center points was conducted and the production of the enzymes was determined at each run as indicated in Table 3. Analysis of variance (ANOVA) of the data in Table 3 has been attained and shown in Table 4. The analysis was carried out independently on each enzyme to identify the model of the highest specialty and fitness to describe the production. The results in Table 4 showed that the production of each enzyme was correlated with the four medium components through a definite model and specific equation describing the production. Regarding the significance and validity, all of the obtained models were statistically significant (p-value less than 0.05) and there were low chances that a "Model F-Value" could occur due to noise. Lack of fit was not significant in all models which implies significant fitness of the models. Signal to noise ratio "Adeq Precision" was considerably greater than 4 in the four models which indicated an adequate signals and validity to use such models to navigate the design space [30]. In case of chitinase and WCL, the type of the model was the same (quadratic model), however the equations describing the two model were completely different. Simple models of the type linear and two-factor interaction (2FI) were obtained in case of protease and glucanase respectively. The linear equation describing the production of protease was simple enough to indicate clear positive effect of all components on the enzyme production. Considering the significant terms in all models, it was obviously revealed that the term A (codes for peptone concentration) was significant only in case of protease production while the term B (codes for chitin concentration) was significant only with chitinase production. In such regard, the role of peptone and chitin in production of protease and chitinase respectively was reported in elsewhere [31,32,33,34,35]. However, the terms C and D (code for baker's yeast and cell wall concentrations respectively) were significant in production of many enzymes. Interaction between variables indicates the ability of any of interacting variables to alter the profile of the effect of the other. Regarding the interactions between the different components, the most common was that between A and D (peptone and cell wall concentration) which was realized in production of chitinase, glucanase, and WCL. Other interactions (between C & D or C & B) were occasionally noticed.

Table 1 Effect of different media on production

Medium type	Enzymatic activity (U/mL)			
	Glucanase	Chitinase	Protease	WCL
M1	1.76	00.15	70.00	2.37
M2	1.38	00.08	54.00	2.18
M3	0.76	00.15	113.00	11.10
M4	20.27	11.07	108.73	15.67

Ability of the four obtained models to predict the productivity of the enzymes was inferred from the relation between prediceted enzyme concentration versus actual concentration, as depicted graphically in Fig. 3. Distribution of the points around the line of the perfect prediction revealed a reliable predictability. The models were thus employed to find the optimum composition of the maximum production. Response surface methodology (RSM) on each of the four models defined the medium composition that was specific for the highest production of the concerned enzyme. Table 5 summarized the composition of a four media basing on different models with expected and verified productivity. As indicated in Table 5, the practical validation of productivity realized high proficiency of BBD and RSM in system prediction and optimization. It was possible to verify the maximum yields of 14.97, 27.89, 137.59 and 65.28 U/ml for chitinase, glucanase, protease and WCL respectively using fermentation media I, II, III and IV specific for each enzyme. Specificity of medium for maximum production of an enzyme varied considerably with slight change in composition as it was inferred from media I and IV.

Table 2 Codes and levels of factors studied by Box-Behnken design and response surface methodology

Factor code	Conc. (%)	Low level (-1)	Mean level (0)	high level (+1)	Std. Dev.
A	Peptone	0	0.05	0.1	0.0333
B	Chitin	0	0.1	0.2	0.0666
C	Baker's yeast	0	0.5	1	0.3333
D	Cell wall	0	0.3	0.6	0.2

Table 3 BBD for studying the production of different enzymes in relation to four main nutritional components

Run	Factor A Peptone (%)	Factor B Chitin (%)	Factor C Baker's yeast (%)	Factor D Cell wall (%)	Response 1 Chitinase (U/ml)	Response 2 Glucanase (U/ml)	Response 3 Protease (U/ml)	Response 4 WCL (U/ml)
1	0	0.1	0	0.3	3.1	17.1	56.8	32.5
2	0.05	0.1	1	0.6	4.4	29.1	137.2	10.0
3	0.05	0	0.5	0	10.0	19.7	75.2	35.0
4	0.05	0.1	0	0.6	7.5	18.7	129.2	41.0
5	0.05	0.2	0.5	0.6	12.0	26.2	130.0	31.0
6	0.05	0.1	0.5	0.3	10.3	22.6	99.0	12.0
7	0.05	0.1	0.5	0.3	9.2	18.6	125.6	18.0
8	0.05	0.1	0.5	0.3	13.7	19.6	101.6	17.0
9	0.05	0.2	0.5	0	12.1	16.9	90.4	45.0
10	0.05	0	1	0.3	2.1	28.2	130.8	20.0
11	0.05	0	0.5	0.6	7.9	20.4	115.6	42.0
12	0	0.1	0.5	0.6	1.7	21.5	130.0	14.5
13	0.1	0.2	0.5	0.3	15.0	22.9	127.6	43.5
14	0.1	0.1	0	0.3	11.6	19.0	115.8	41.0
15	0	0.1	1	0.3	4.7	21.4	90.0	37.0
16	0.1	0.1	0.5	0	3.3	21.0	123.6	31.0
17	0.05	0.2	1	0.3	8.5	22.1	129.8	29.0
18	0.1	0.1	1	0.3	6.0	23.0	123.2	41.5
19	0.05	0.2	0	0.3	7.1	15.0	80.6	40.1
20	0	0	0.5	0.3	11.9	21.4	103.2	58.0
21	0	0.2	0.5	0.3	15.8	17.9	101.8	63.0
22	0.05	0.1	1	0	1.7	21.6	116.8	40.5
23	0.05	0.1	0	0	7.4	7.2	64.2	35.0
24	0.05	0	0	0.3	4.9	9.1	80.0	61.0
25	0.1	0	0.5	0.3	9.1	18.9	122.8	69.0
26	0	0.1	0.5	0	12.0	10.8	93.2	63.0
27	0.1	0.1	0.5	0.6	11.7	19.5	132.4	51.0

Table 4 ANOVA analysis for modelling the production of different enzymes

Model specification	Chitinase	Glucanase	Protease	WCL
Type of model	Quadratic	Two-Factor Interaction (2FI)	Linear	Quadratic

p-value	0.0102	< 0.0001	< 0.0001	0.0016
Significance and validity of model	Significant. There is only a 1.02% chance that a "Model F-Value" could occur due to noise.	Significant. There is only a 0.01% chance that a "Model F-Value" could occur due to noise.	Significant. There is only a 0.01% chance that a "Model F-Value" could occur due to noise.	Significant. There is only a 0.16% chance that a "Model F-Value" could occur due to noise.
Lack of Fit	0.5332 (not significant)	0.4684 (not significant)	0.6761 (not significant)	0.1180 (not significant)
Fitness parameters				
R^2	0.8248	0.8432	0.7039	0.8783
Adj. R^2	0.6205	0.7453	0.6501	0.7364
Signal/noise ratio	6.640	10.505	11.788	9.351
Significant terms	B, C^2, AD	C, D, AD, BC	A, C, D	C, AD, CD, A^2, B^2
Factors showing interaction	A with D	A with D B with C	No interactions	A with D C with D
Model equation	Chitinase activity = +9.05617 -43.1346 * A -20.65496 * B +17.47349 * C -6.05064 * D +96.08375 * A * B -72.22092 * A * C +312.55 * A * D +21.19924 * B * C +16.95804 * B * D +4.21633 * C * D -115.26529 * A^2 +103.29211 * B^2 -19.63679 * C^2 -22.92389 * D^2	Glucanase activity = +6.79894 +49.13167 * A -6.8325 * B +18.1425 * C +17.2425 * D +375.975 * A * B -2.81* A * C -205.2 * A * D -60.705 * B * C +70.5375 * B * D -6.84 * C * D	Protease activity = +57.11852 +284 * A +27.16667 * B +33.53333 * C +58.61111 * D	Cell wall lytic enzymes activity = +80.23333 -942.33333 * A -346.5 * B -27.33333 * C -68.72222 * D -1525 * A * B -40 * A * C +1141.667 * A * D +149.5 * B * C -175 * B * D -60.83333 * C * D +7873.33333 * A^2 +1863.33333 * B^2 +20.53333 * C^2 +71.48148 * D^2

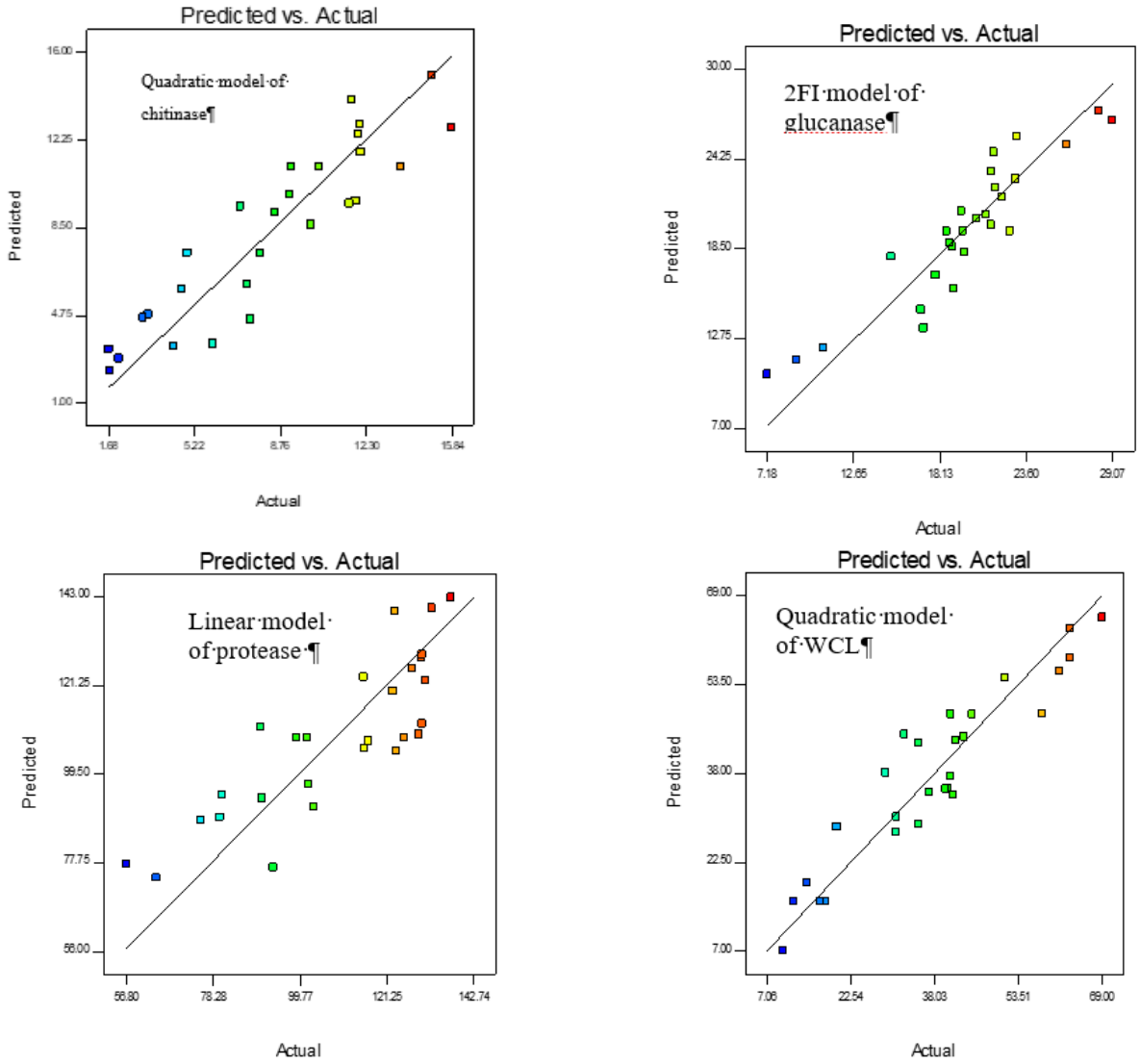

Fig. 3.Relation between prediceted enzyme concentration versus actual concentration for the models describing production of different enzymes

Table 5 Composition of the four media developed on BBD and RSM for highest production of each enzyme with predicted and verified productivity

		Medium I	Medium II	Medium III	Medium IV
Model used to develop the medium		Quadratic model of chitinase	2FI model of glucanase	Linear model of protease	Quadratic model of WCL
Structure of medium*	**Baker's yeast conc. (%)**	0.33	0.91	0.77	0.38
	Chitin conc. (%)	0.20	0.00	0.13	0.01
	Cell wall conc. (%)	0.45	0.51	0.52	0.45
	Peptone conc. (%)	0.10	0.00	0.08	0.10
Enzyme of highest production, the expected yield		Chitinase, 16.93 U/ml	Glucanase, 28.91 U/ml	Protease, 138.43 U/ml	WCL, 70.85 U/ml
The yield as practically validated		14.97 U/ml	27.89 U/ml	137.59 U/ml	65.28 U/ml

* The structure concerned with the main components targeted through BBD and RSM. Other medium components were as follow (%): K_2HPO_4: 0.3, KH_2PO_4; 0.2, $MgSO_4$. $7H_2O$: 0.03, Glucose: 0.02 and $NaNO_3$: 0.1.

3.5. Correlation between productivities of different hydrolytic enzymes

The possibility to potentiate the production of more than one enzyme to the highest yield simultaneously (in the same fermentation medium) was realized through degree of correlation between their productivities; the enzymes whose concentrations increase or decrease together are of a positive correlation and more likely to be simultaneously enhanced to highest yield in the same fermentation medium. Pearson's correlation coefficient (r) was calculated for the relation between productions of different enzymes in 27 runs of BBD. As shown in Table 6, there was moderate positive correlation between glucanase and protease production (r= 0.69), and with less extent was the relation of chitinase with WCL (r= 0. 35). Accordingly, it was thought that improving the yield of glucanase moves with good extent in the same direction with increase in protease concentrations, and it is nearly the same case between chitinase and WCL. The results also revealed negative correlation with different degrees between production of some enzymes. The highest negative correlation has appeared between glucanase and WCL (r= -0.49) indicating that increasing the yield of one of them moves with considerable degree in the direction of decreasing the yield of the other which may reflect an inhibitory metabolic mechanisms between such enzymes according to carbon metabolite repression between their inducing substrates [36].

Table 6 Pearson's correlation coefficient (r) for the relation between production of different enzymes

	Chitinase	Glucanase	Protease	WCL
Chitinase	1	-0.14	-0.01	0.35
Glucanase	-0.14	1	0.69	-0.49
Protease	-0.01	0.69	1	-0.21
WCL	0.35	-0.49	-0.21	1

3.6. Coproduction of hydrolytic enzymes

From the preceding data and analysis, it was inferred that each enzyme has its specific favorable requirements that have not potentiated the production of other enzymes by the same extent. Finding a medium that supports the coproduction of all enzymes simultaneously as much as possible and comparing production of each enzyme in such medium with its productivity in its specific optimized medium is the target adopted here. Basing on BBD and RSM, the composition of the main components of the best suited medium for coproduction was identified. The composition of the medium, predicted and verified productivity were presented in Table 7. The medium could potentiate the productivity to 12.05, 23.08, 130.90 and 57.00 U/ml for chitinase, glucanase, protease and WCL respectively which were equivalent to 80.51%, 82.73%, 95.14% and 87.32% of the maximum yield of each enzyme obtained in media I, II, III and IV on respective order. Superiority of coproduction medium was clearly demonstrated when testing suitability of other media (I, II, III and IV) in coproduction. Each of the four media was evaluated for suitability in coproduction by determining the concentration of all enzymes and expressing them as a percentage of the maximum yield obtained for each enzyme in its specifically optimized medium, as indicated in Table 8. Though each medium showed highest specificity for maximum production of an enzyme, the medium was of significant poor specificity for some other enzymes whose productivities had significantly dropped to less than 50%. The success in broadening the specificity of the medium, to fulfill the favorable production of all enzymes without uncontrolled drop in each of their yields, clarified the unique surprising privilege of statistical modeling in fermentation technology. Moreover, the ability to produce several products simultaneously with more than 80% of their maximum yields, even there were some considerable negative correlations between their production, represented a valuable record in fermentation technology and was thought to have deep future implications concerning the financial returns of fermentations.

Table 7 Composition, predicted and verified productivity of the optimum medium for coproduction of all enzymes

Composition of the medium (main components %)			
Baker's yeast	0.21		
Chitin	0.2		
Cell wall	0.6		
Peptone	0.1		
Amount of produced enzymes			
	Predicted (U/ml)	**Practically validated amounts**	
		U/ml	**% of maximum yield***
Chitinase	17.12	12.05	80.51
Glucanase	24.70	23.08	82.73
Protease	133.13	130.90	95.14
WCL	64.44	57.00	87.32

* Maximum yield obtained of chitinase was 14.97 U/ml that was obtained in medium I; Maximum glucanase was 27.89 U/ml that obtained in medium II; maximum protease was 137.59 U/ml that obtained in medium III;Maximum WCL was 65.28 U/ml that obtained in medium IV.

3.7. Investigation of antifungal activity

The crude culture filtrate of *Streptomyces rochei*-obtained from the final optimized medium which containing the highest richness of all enzymes (in simultaneous existence) had been employed in *in-vitro* challenge against some pathogenic fungi where intensive antifungal activity was realized with *F. graminearum*(20 mm), *M. racemosus* (13 mm), *F. solani*(20 mm), and *C. albicans*(25 mm). Similarly,Kumar et al. [37] obtained an antagonistic activity of *Paenibacilluselgii* strain HOA73 against phyto-

pathogenic fungi and human fungal pathogens. Also, Philip et al. [38] reported the antagonistic activity against *Candida albicans*, *Aspergillus niger*, *Microsporum gypseum*, *Trichophyton rubrum*and *Saccharomyces cerevisiae* through the production of hydrolytic enzymes. Therefore, the produced filtrate can be used for control of pathogenic fungi.

Table 8 Suitability of the different media for the coproduction of all enzymes

Productivity %* of the different enzymes in the four media			
Medium I	**Medium II**	**Medium III**	**Medium IV**
100% Chitinase	100% Glucanase	100% Protease	100% WCL
67.99% Glucanase	51.14% Chitinase	63.80% Chitinase	72.41% Chitinase
85.81% Protease	84.78% Protease	89.21% Glucanase	68.35% Glucanase
84 % WCL	57.12% WCL	26.88% WCL	74.42% Protease

*Expressed as a percentage of enzyme concentration to the maximum amount obtained; chitinase activity was expressed as percentage of 14.97 U/ml; glucanase activity was a percentage of 27.89 U/ml; protease activity was a percentage of 137.59 U/ml; lytic activity was a percentage of 65.28 U/ml.

3.8. Study of mode of action by testing cell wall integrity

In the present investigation, *C. albicans* was selected as a target model microbe to study the effect of optimized culture filtrate rich in hydrolytic enzymes from the strain MZ227230on the integrity of cell wall. The release of *C. albicans* cell constituents under the effect of different concentrations (100, 75, 50 and 25%) of crude lytic enzymes was investigated to obtain further insight about their mode of action against that pathogen (Fig. 4). The amount of DNA, RNA, and protein released from the cell to the outside medium was dependent on time and enzyme-dose [39]. Where, the maximum amount of leaked constituents reached after 6 h at the concentration of 100% (207 ng/µl DNA, 129 ng/µl RNA and 3.3 mg/ µl protein) compared to the untreated control (3.6 ng/µl DNA, 2.1 ng/µl RNA and 0.28 mg/ µl protein).

These results indicated that lytic enzymes (protease, glucanase and chitinase) acted synergistically to digest the cell wall of *C. albicans*[1]and this effect started with protease which cleave cell wall protein and open it to expose the inner layer of glucan and chitin to be attacked by glucanase and chitinase forming a big hole in the cell wall giving a protoplast which can be lyses immediacy releasing the cell constituents [3]. Thus this multi-enzyme system can be applied for biological control [4,40].

Conclusion

This study is strongly proved that despite of the simultaneous optimized production of multi-targeted enzymes is a hard challenge, when the maximum yield of each product was targeted in the same fermentation medium; the final results revealed that it is possible to obtain about 80% of the maximum yield of each enzyme at the same time in one fermentation medium. And this also could strongly potentiate the antifungal activity of the produced enzymatic system. Accordingly, this will encourage the economic feasibility of the application of one-step fermentation of different multi-targeted products.

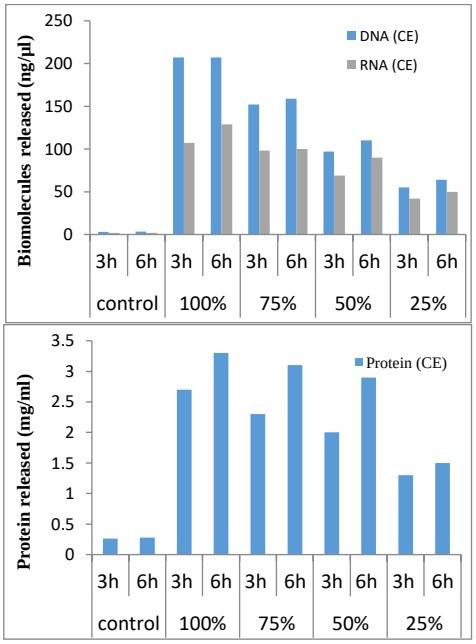

Fig. 4. Quantity of released biomolecules from *C. albicans*ATCC10231 treated with crude culture filtrate (CE)

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Author contribution

Eman W. Elgammal: Conceptualization, Formal analysis, Methodology, Resources, Writing - original draft, Writing - review and editing. Mohamed M. Abdel Aziz: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Writing - original draft, Writing - review & editing and Software. Eman F. Ahmed: Conceptualization, Methodology, Resources, review and editing.

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