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Comparison of Optimal Fungal Pectinase Activities Using the Box-Behnken Design

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ABSTRACT

Pectinases catalyze pectin via hydrolysis and de-esterification to simpler forms. Pectinase produced by the fermentation process can be enhanced through optimization. The Design-Expert Software (package) file version 13.0.3 was used to predict the optimal activity of the enzyme with *Citrus sinensis* (orange) peels, *Triticum aestivum* (wheat) bran, and *Thaumatococcus danielli* (Miraculous berry) fruit peels as substrates, while *Aspergillus niger*, *Penicillium sp.* and *Pichia kudriavzevii* strain F2-T429-5 as microbes for the solid-state fermentation process. The microorganisms were selected based on their zones of hydrolysis of pectin. The Box-Behnken design was used to generate the experiment runs and analyses of the data obtained from the solid-state fermentation process. The following conditions for optimal pectinase activity were considered; fermentation duration, pH, temperature, particle size, inoculum volume, and agitation during enzyme extraction. *Aspergillus niger* in *Citrus sinensis* peel fermentation resulted in the best enzyme activity of 5.02 U/mL in approximately 6 days of fermentation, pH 4, at 21^{o}C, 0.06-inch substrate size, 1 mL inoculum, and agitation duration during pectinase extraction in approximately 11 min. Pectinase activity with *T. danielli* fruit wastes showed 59.36% decrease relative to *C. sinensis* peel. *A. niger* and *Penicillium sp.* produced a more active enzyme (74.02% and 71.81% respectively) than the newly investigated yeast *P. kudriavzevii* (F2-T429-5) in *T. danielli* fruit wastes fermentation, a vital contribution to mycology. Nevertheless, *Thaumatococcus danielli* and *P. kudriavzevii* (F2-T429-5) employed in the study have good prospects for pectinase production.

Keywords: *Aspergillus niger*, Optimization, *Penicillium*, *Pichia kudriavzevii*, *Thaumatococcus danielli*

Introduction

Pectinases are enzymes that catalyze pectin by hydrolysis and de-esterification to its simpler units.[1,2] Pectinase is reported to make up about 25 % of the global food market for enzymes, hence its importance.[3,4] The importance of this class of enzymes is mainly due to its benefits in the food industry[1], in textile, treatment of industrial wastes, oil extraction, among other areas.[3,5,6] The significance of pectinase is in its degradation of agro wastes containing pectin, a component of the plant cell wall agricultural wastes; as pectinase and cellulase have been used singly and in combination for tomato waste treatment.[7,8]

Various microorganisms such as bacteria and fungi have been reported to produce pectinase.[9,10,11] Also, many substrates have been employed for pectinase production, with variations in the activities of the enzyme produced.[10,12,13] Pectinolytic fungi in controlled environments are easy to grow; hence, their application in large-scale production of the enzyme.[14] Pectinase optimizations have been undertaken in many studies by using one factor at a time (OFAT) and the Response Surface Methodology (RSM) methods.[10,15] Pectinase optimization processes are undertaken to improve the activity of the enzyme[1,10,16]

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The OFAT method of optimization is undertaken by varying one of the conditions for pectinase production while keeping the other conditions constant (also known as the 'one variable at a time' method).[10] The RSM involves mathematical, statistical, and computer programming in arriving at the conditions for the best productivity of the enzyme.[16-18] Hence, this model predicts the dependent variables from the analyses of the interactions among the independent variables.[16] The enhancement of the productivity of the enzyme can be obtained with both the OFAT and RSM. However, the use of one factor at a time (OFAT) method of optimization does not adequately account for the interactions that occur during pectinase production; hence, improvement of the process through the use of the Response Surface Methodology (RSM). The RSM has been used to improve the media for pectinase production.[19] Generally, RSM modeling has been applied in the production of cost-effective pectin enzymes.[19] The Box-Behnken Design (BBD) is among the tools/instruments in the RSM; hence, its utilization as the number of experimental runs are fewer than the Central Composite Design (CCD). However, both CCD and BBD are used to improve enzyme production conditions in both downstream and upstream processes.[20-22] The BBD and CCD have been shown to increase enzyme production with minimal cost.[23]

Therefore, BBD was used to predict the optimal pectinase activity from selected agricultural wastes and fungi combinations. These fungi require optimization because, in their wild states, the production of the pectinase is relatively low.[15] Optimization of conditions for pectinase production has been proven to improve the enzyme secreted by microorganisms.[19,23] The conditions selected for optimization were as in the previous study using the OFAT approach[10] with a few additional conditions in the current study. Additionally, BBD in this study was used to predict the optimal pectinase (pectin enzyme) activity. The new strain of the fungus, *Pichia kudriavzevii*, and substrate, the *Thaumatococcus danielli* plant fruit peels, were also studied for pectinase production.

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Materials and Methods

Collection of sample

The substrates (*Citrus sinensis* peel and *Triticum aestivum* bran) used for fermentation were sourced (April 2019) from the Nigerian Army Arena, Oshodi, Lagos State, Nigeria. The sweet prayer plant (*Thaumatococcus danielli*) fruits were sourced from the Owode-Yewa forest plants area, Atan-Ota (September 2019) in Ogun State, Nigeria, before using the fruit peels in the fermentation process. The substrates were identified by botanist, in the Pulp and Paper Division of Federal Institute of Industrial Research, Oshodi, Lagos State, Nigeria.

Microorganisms and screening for pectinase production

The fungi (*Aspergillus niger, Penicillium sp., Pichia kudriavzevii* strain F2-T429-5, *Pichia kudriavzevii* strains *F2-T429-5* and *CY902*, and *Saccharomyces cerevisiae*) were isolated from deteriorating fruit wastes (Table 2). The fungi isolates were selected for the study due to their highest zones of clearance for pectinase production. The 'deteriorated' fruit wastes consisted of banana, orange, pineapple, corn cobs (5 g each), and accompanying soil samples at refuse dumpsites at the Federal Institute of Industrial Research, Oshodi, (FIIRO) Lagos State, Nigeria. The fungi were isolated by using the pour plated method.[24] Serial dilutions were undertaken, in that 1 g of each of the deteriorated fruit samples were ground and diluted in 9 mL of distilled water in sterile test tubes and homogenized for use as stock solution. After which, ten-fold serial dilutions of the stock solutions obtained from each of the deteriorated fruit samples were carried out with distilled water as the diluent. The fungal growth in Simple Potato Dextrose Agar (PDA) medium (prepared using the manufacturer's instructions) with streptomycin antibiotic (2.0 mg/L) added into the potato dextrose agar (PDA) medium to eliminate the growth of bacteria in the medium, and autoclaved at 121^{o}C for 15 minutes. Each fungus was inoculated and incubated at 25 ± 3^{o}C for five days. The subculture of each of the several colonies formed was then carried out to obtain distinct colonies. The yeast and moulds were identified using protocols previously described by other authors.[25,26]

Thereafter, each isolated fungi was screened for pectinolytic activity using a modified Czapek medium (substituting pectin for cellulose).[27] The zones of hydrolysis obtained indicated a measure for the extent of pectinase production for each isolate and used as a condition for inclusion in the study. After preparing the modified Czapek media, sterilization in an autoclave (for 15 min at 121^{o}C), a sterile cork borer was used to drill a circle in the center of the modified Czapek media plate, each of the pure fungal isolates (10.46×10^6 cells/mL and 10^6 mL for the moulds washed off from Petri dishes using sterile distilled water containing three drops of Tween 80 surfactants) were inoculated into each of the spots on the plates created by the cork borer. Incubation of the plates at 25 ± 3^{o}C for 48 h was undertaken. The hydrolysis zone (clearance) obtained (Table 2) indicated the pectinase secretion ability of each microbe. After that, these plates were then floored with potassium iodide (KI) solution to enhance the visibility of the hydrolytic portions on the media for measurement in millimeters using a simple millimeter rule (Table 2).[28] However, for this study, two moulds and one yeast strain of the fungal microbes isolated were selected (Table 2) and identified for the optimization process.[25,26]

Pectinase production

Solid-state fermentation was undertaken with 2.0 g of each of the substrates. The fruits were washed, peeled, and the peels oven-dried for 72 h at 58^{o}C± 3^{o}C; and milled (with a Hammer Mill) to particle sizes as in Table 1. Each of the weighed substrates was transferred into labeled Erlenmeyer flasks (250 mL) flasks. A 2.0 mL solution of the nutrient medium was added to each of the flasks. The nutrient medium constituted KH_2PO_4-0.12g, KNO_3-0.12g, K_2HPO_4-0.12 g, and $MgSO_4$-0.02 g (diluted with distilled water made up to 200 mL); pH adjustments were done using 1M HCl and NaOH.

Table 1: Generation of the experimental runs using the Box-Behnken model for the prediction of the optimal pectinase activities from six conditions for pectinase production

S/No.	A	B	C	D	E	F
1	7	4.5	35	0.027	5	35
2	7	2	35	0.027	3	35
3	4	4.5	50	0.027	3	10
4	4	7	35	0.062	1	10
5	4	4.5	35	0.062	3	35
6	4	7	35	0.062	5	10
7	4	2	20	0.062	5	35
8	1	4.5	35	0.097	1	35
9	4	4.5	50	0.097	3	60
10	4	4.5	20	0.027	3	60
11	4	4.5	35	0.062	3	35
12	4	4.5	20	0.027	3	10
13	4	4.5	35	0.062	3	35
14	4	2	50	0.062	1	35
15	7	4.5	50	0.062	3	10
16	4	7	20	0.062	5	35
17	4	4.5	20	0.097	3	10
18	4	7	20	0.062	1	35
19	7	4.5	35	0.097	1	35
20	4	4.5	20	0.097	3	60
21	7	2	35	0.097	3	35
22	4	2	35	0.062	5	60
23	4	4.5	50	0.027	3	60
24	1	2	35	0.097	3	35
25	7	7	35	0.097	3	35
26	7	4.5	35	0.027	1	35
27	7	4.5	20	0.062	3	60
28	7	4.5	20	0.062	3	10
29	4	2	35	0.062	1	60
30	7	7	35	0.027	3	35
31	4	2	35	0.062	1	10
32	7	4.5	50	0.062	3	60
33	1	4.5	20	0.062	3	60
34	1	4.5	50	0.062	3	10
35	1	4.5	35	0.027	5	35
36	1	7	35	0.027	3	35
37	4	4.5	35	0.062	3	35
38	4	4.5	50	0.097	3	10
39	1	4.5	35	0.097	5	35
40	4	2	50	0.062	5	35
41	1	4.5	35	0.027	1	35
42	4	2	20	0.062	1	35
43	4	7	35	0.062	5	60
44	4	2	35	0.062	5	10
45	4	7	35	0.062	1	60
46	1	4.5	50	0.062	3	60
47	4	7	50	0.062	1	35
48	4	4.5	35	0.062	3	35
49	7	4.5	35	0.097	5	35
50	1	7	35	0.097	3	35
51	4	4.5	35	0.062	3	35
52	4	7	50	0.062	5	35
53	1	2	35	0.027	3	35
54	1	4.5	20	0.062	3	10

A - fermentation duration (d); B - pH; C - temperature (^{o}C); D - particle size (inch); E - inoculum volume (mL) and F - agitation (min)

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Table 2: Fungal Screening for pectinase production

Fungi	Source	Hydrolysis of the pectin	Zone of hydrolysis (mm)
Aspergillus niger (mould)	Deteriorated corn cobs and soil samples	+	83.5 ± 0.07
Penicillium sp. (mould)	Deteriorated corn cobs and banana	+	72 ± 0.09
Pichia kudriavzevii strain *F2-T429-5* (yeast)	Deteriorated orange and pineapple	+	37 ± 0.12
Pichia kudriavzevii strain CY902 (yeast)	Deteriorated orange and pineapple	+	34 ± 0.25
Saccharomyces cerevisiae (yeast)	Deteriorated orange and pineapple	+	31 ± 0.17

Mean ± standard deviation

Thereafter, the contents were autoclaved and cooled to room temperature before aseptically adding 1% (0.02 g) of citrus pectin (Sigma Aldrich) as an inducer to the contents.[10] Each microorganism was inoculated into labeled sterile media and incubated for the durations in Table 1. It should be noted that, the freshly cultured yeast cells and spores of fungi were washed off from Petri dishes using sterile distilled water containing three drops of tween 80 surfactants. The inoculum concentration of 10.46×10^6 cells/mL was determined spectrophotometrically with variations in volume as in Table 1; and, 10^6 mL for the moulds using a hemocytometer. 0.1 M NaCl was used as the extraction solvent for the enzyme from the fermented substrates.[10]

Assay for pectinase activity

The procedure by Miller,[29] was employed with modifications, as 3 mL of 1% citrus pectin solution prepared by dilution in distilled water and homogenized. One millilitre (1 mL) of the crude pectin enzyme (supernatant) was added, and the mixture was incubated at 50°C in a water bath for 10 min. After which, one mL of freshly prepared dinitrosalycyclic acid (DNSA) solution was added and boiled for 5 min and, after that, left on the laboratory bench to cool to room temperature. UV-Vis spectrophotometer was preset at 540 nm. The readings from the spectrophotometer were read as absorbance. Thereafter, the absorbances converted to pectinase activities using the standard curve obtained from galacturonic acid.[10,29] Prediction of the treatment conditions for optimum pectinase activity was obtained using the Box-Behnken design in the Design-Expert software package file version 13.0.3. Random generation of experimental runs (Table 1) in triplicates using the quadratic model for optimal pectinase activity prediction and analysis of variance for the model obtained in the same software package. Six variable conditions were screened namely: fermentation duration (days), 1 to 7 d; pH, 2 - 7; temperature, 20 - 50°C; particle size for the substrates, 0.027 - 0.097 inches; inoculum volume, 1 - 5 mL and agitation of fermented substrates before filtration in 10 - 60 min.

Results and Discussion

The choice of Box-Behnken Design (Table 1) in this study for prediction of optimal pectinase activity was because it showed as the experimental design for RSM with fewer experimental runs (for each replicate) relative to the central composite design (Charts 1 and 2). Hence, its advantage is in reducing the cost (financial implication) associated with this study. The Box-Behnken design was used in the study to determine the interactions amongst the independent variables (factors) investigated for the prediction of the optimum activity of the pectinase produced.

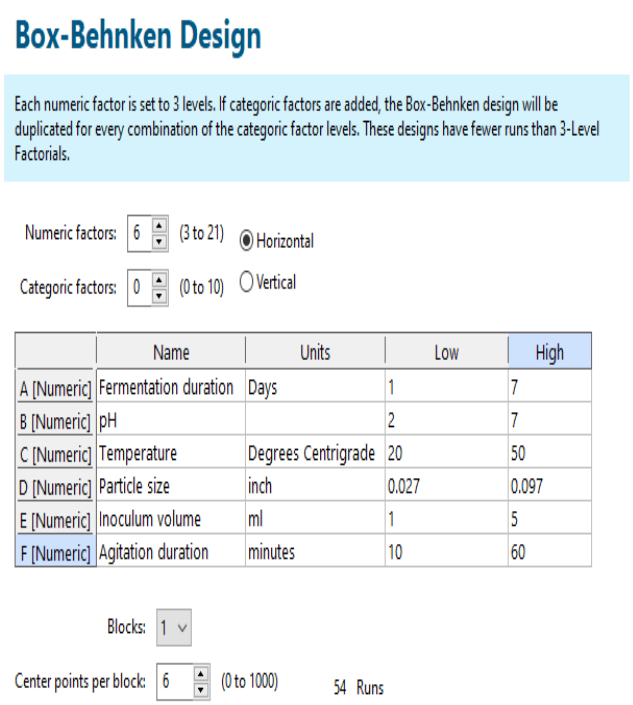

Chart 1: The Box-Behnken Design showing 54 runs for the design of the experiment

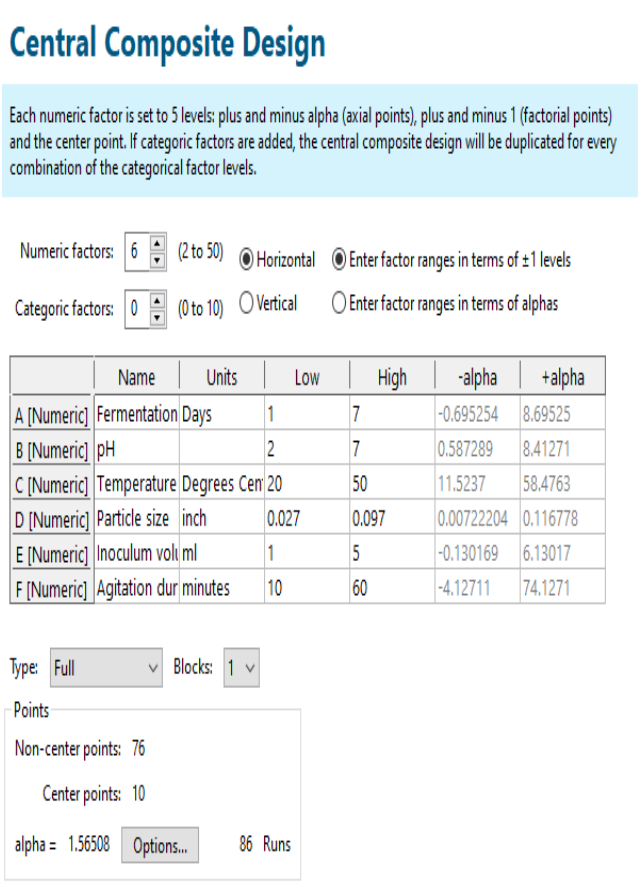

Chart 2: The Central Composite Design showing 86 runs for the design of the experiment

The independent factors investigated were fermentation duration (days), A; pH, B; temperature, C; particle size, D; inoculum volume, E; and, duration of agitation for pectinase extraction, F. Each of the

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six independent variables studied at three different levels are shown in Table 1. The three levels indicated the low and high levels, with the middle level, the average for the low and high levels. The levels generated by the software were, A (1, 4 and 7 d), B (2, 4.5 and 7), C (20, 35, and 50 ^{o}C), D (0.027, 0.062, and 0.097 inches), E (1, 3, and 5 mL) and F (10, 35, and 60 minutes) (Table 1). The Box-Behnken Design had a total of 54 experimental runs (Chart 1; Table 1). However, these 54 runs were done in triplicates (162 runs) for each fungus and substrate combination, with the results displayed graphically in Figures 1-9. The predicted maximum pectinase activity of 5.02 U/mL based on the responses of the data from the experimental runs (Table 1) was obtained with *A. niger* in *Citrus sinensis* peels as substrates in approximately the 6th day of fermentation, pH of approximately 3.9 with the parameters as indicated in the actual factors in Figure 1. Therefore, the result depicts *Citrus sinensis* peel as the substrate with the most pectin content; since pectin is the substrate for pectinase production. Hence, for pectinase to be produced, the *Citrus sinensis* peel would have influenced the microbial release of the enzyme.[10] In a related study, orange peel and wheat bran induced the maximum production of the enzyme using the quadratic model of the central composite design for prediction of the response.[30] *A. niger* also showed the largest hydrolysis zone of pectin (Table 1); hence, its use in literature for commercial pectinase production.[7,31] Generally, the predicted R^2 and adjusted R^2 values were in a reasonable agreement as adjudged by the Design-Expert software package file version 13.0.3 used for analysis; hence, making the results reliable. The predicted R^2 and adjusted R^2 values were 0.9994 and 0.9992 (Figure 1), 0.9771 and 0.9904 (Figure 2), 0.9354 and 0.9747 (Figure 3), 0.9997 and 0.9999 (Figure 4), 0.8171 and 0.9286 (Figure 5), 0.9578 and 0.9798 (Figure 7), 0.7884 and 0.9150 (Figure 8), and 0.8236 and 0.9308 (Figure 9). Except for Fig. 6 (-0.2543 and 0.4341), which implied that the overall mean (1.71) may be a better predictor of the response than the current model used in the study. In addition, though the adequate precision of 5.4229 (Figure 6) showed that the model could be used to navigate the design space; however, a higher-order model may also predict better activity. Pradhan and colleagues used an RSM second-order polynomial model for the prediction of optimal output.[16] Hence, justifying the need for employing a higher-order model for prediction of the optimal activity in Figure 6.[16] The lack of fits which were not significant in the analyses indicated each extent of non-significance relative to pure error, suggesting that there is a possibility for the lack of fits occurring due to noise. Hence, making the model used for the study good for the design and analysis of the data. It is important to note that, the quadratic model was used for generation of all the experimental runs and subsequent analysis of the data obtained, which was the basis for comparison of the results. The means to the standard deviations from the data obtained after undertaking the solid-state fermentation for production of the enzyme were as follows; 4.90 ± 0.0107 (Fig. 1), 4.21 ± 0.0251 (Fig. 2), 2.38 ± 0.0181 (Fig. 3), 4.12 ± 0.0026 (Fig. 4), 3.74 ± 0.0594 (Fig. 5), 1.71 ± 0.1459 (Fig. 6), 1.77 ± 0.0271 (Fig. 7), 1.64 ± 0.0616 (Fig. 8) and 0.4535 ± 0.0201 (Fig. 9). For cases of significant lack of fits, the adequate precisions (in the Fit Statistics), indicating the measures of each signal to noise ratio greater than 4 (31.82, 17.80, 17.47, and 21.17 for Figs. 3, 5, 8 and 9 respectively) were used to indicate adequate signals; serving as a basis for the inclusion of the quadratic model in the study.

The approximate predicted optima pectinase activities were as follows; 4.60 U/mL (5 d and pH of 4.6) for *Penicillium sp.* in *Citrus sinensis* peel fermentation (Fig. 2), 2.48 U/mL (5 d and, pH of 4.4) for *Pichia kudriavzevii* strain *F2-T429-5* in *Citrus sinensis* peel fermentation (Fig. 3), 4.38 U/mL (6 d and pH of 4.6) for *Aspergillus niger* in *Triticum aestivum* bran (Fig. 4), 4.10 U/mL (5 d and pH of 4.5) for *Penicillium sp.* in *Triticum aestivum* bran fermentation (Fig. 5), 1.89 U/mL (5 d and pH of 4.8) for *Pichia kudriavzevii* (strain F2-T429-5) in *Triticum aestivum* bran fermentation **(**Fig. 6). The predicted optima activities for *Aspergillus niger* (Fig. 7), *Penicillium sp.* (Fig. 8), and *Pichia kudriavzevii* strain *F2-T429-5* (Fig. 9) in *Thaumatococcus danielli* plants were 2.04 U/mL (5 d and pH 4.8), 1.88 U/mL (6 d and pH 5.7), 1.88 U/mL (5 d and pH of 6), with the least activity obtained at 0.53 U/mL (5 d at pH of 4) respectively.

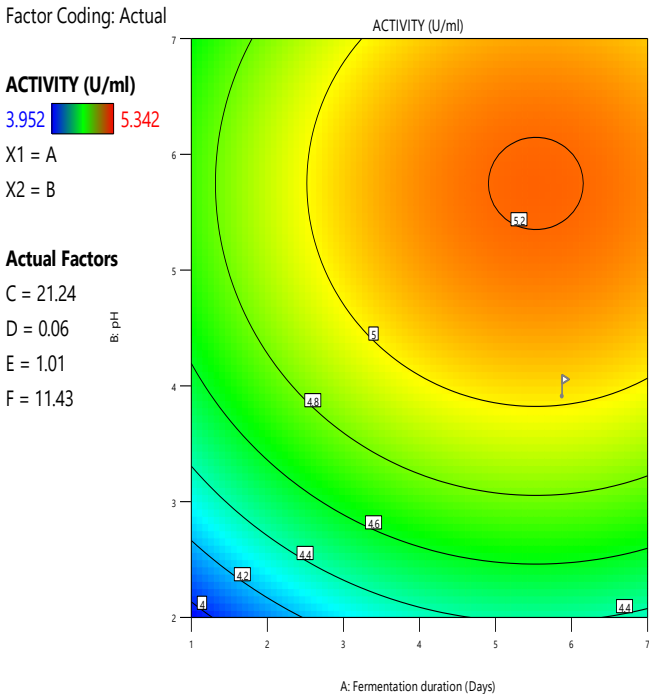

Figure 1: Effect of fermentation duration and pH on pectinase activity with *Aspergillus niger* and *Citrus sinensis* peels as substrate.
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

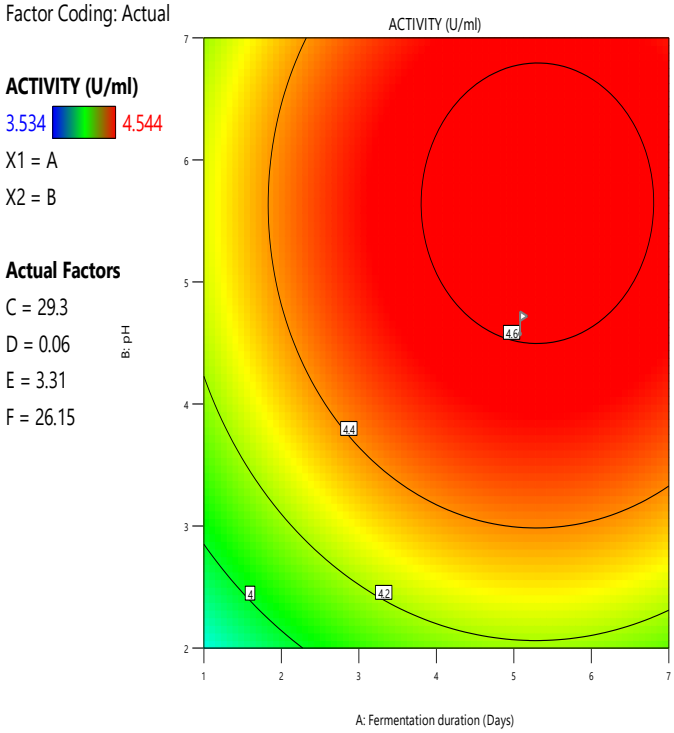

Figure 2: Effect of fermentation duration and pH on pectinase activity with *Penicillium sp.* and *Citrus sinensis* peels as substrate.
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

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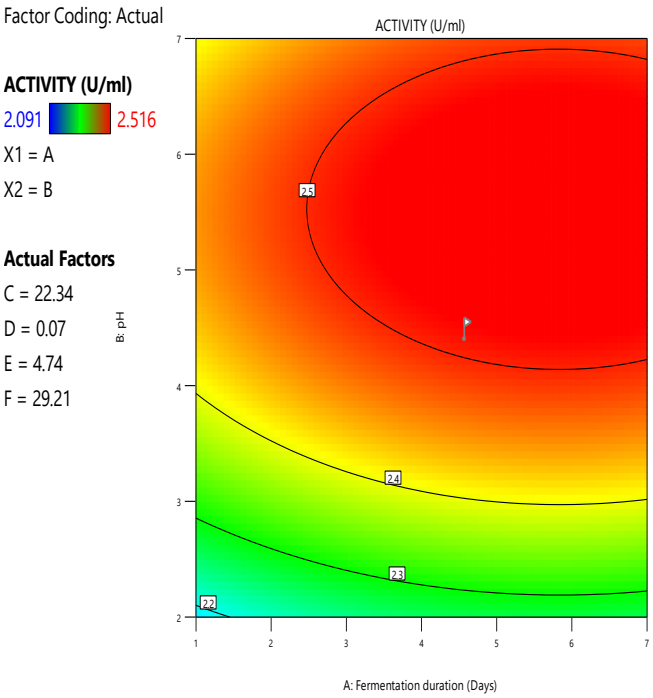

Figure 3: Effect of fermentation duration and pH on pectinase activity with *Pichia kudriavzevii* strain F2-T429-5 and *Citrus sinensis* peels as substrate.
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

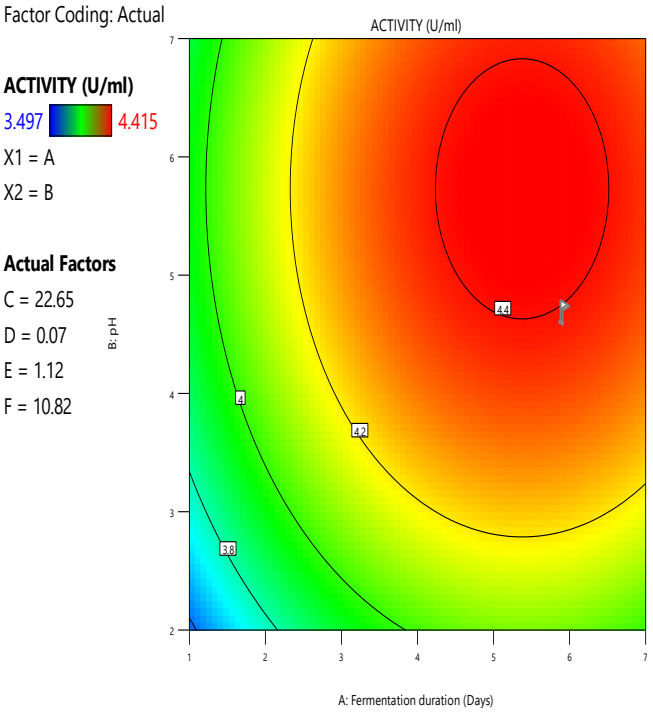

Figure 4: Effect of fermentation duration and pH on pectinase activity with *Aspergillus niger* with *Triticum aestivum* bran as substrate.
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

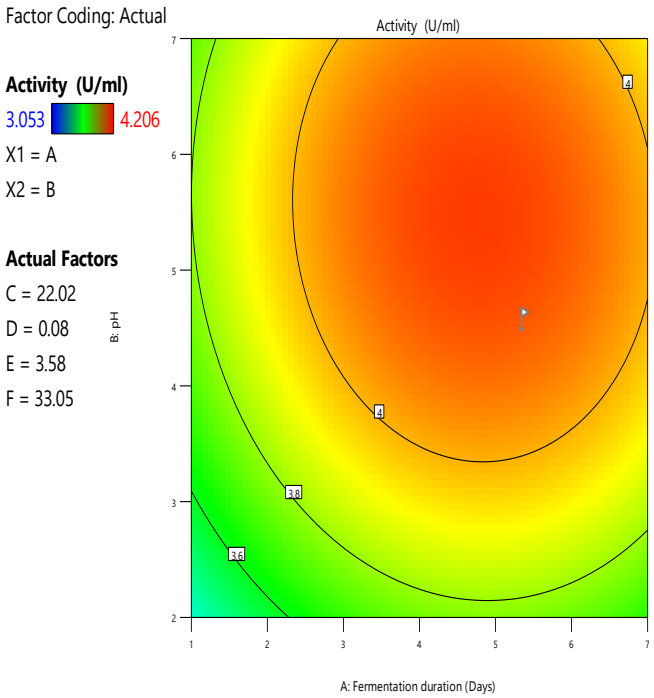

Figure 5: Effect of fermentation duration and pH on pectinase activity with *Penicillium sp*. and *Triticum aestivum* bran as substrate
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

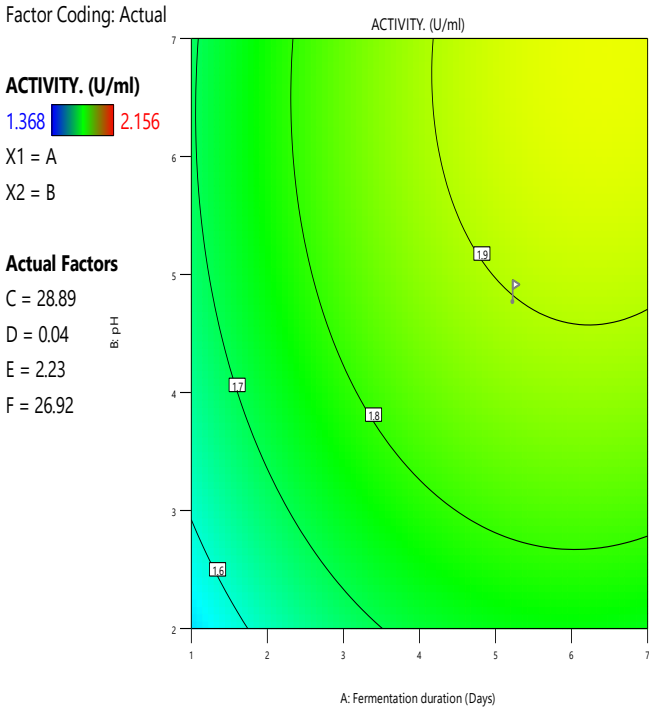

Figure 6: Effect of fermentation duration and pH on pectinase activity with *Pichia kudriavzevii* (strain F2-T429-5) with *Triticum aestivum* bran as substrate
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

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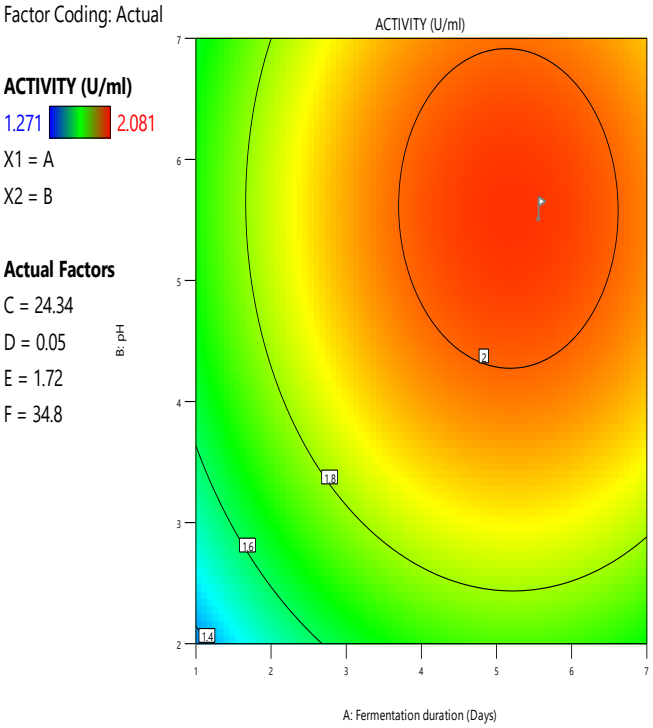

Figure 7: Effect of fermentation duration and pH on pectinase activity with *Aspergillus niger* and *Thaumatococcus danielli* fruit peel as the substrate
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

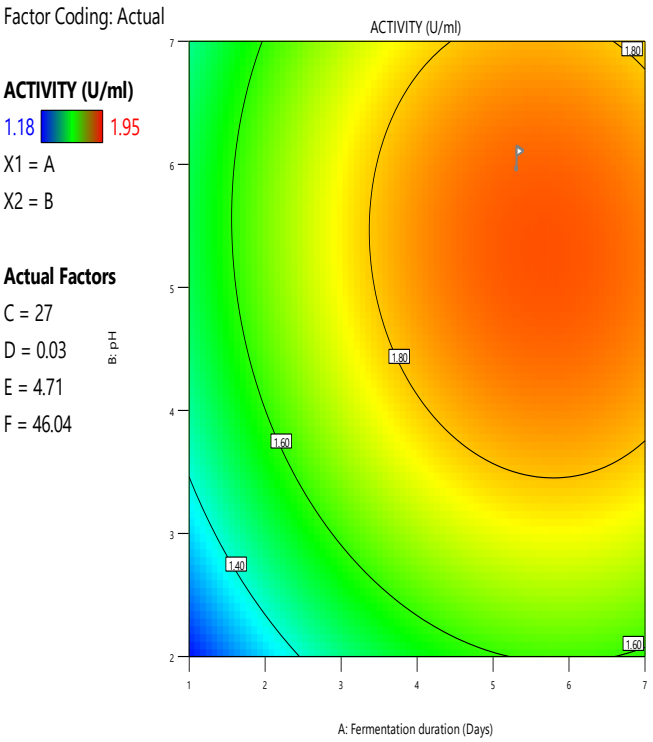

Figure 8: Effect of fermentation duration and pH on pectinase activity with *Penicillium sp*. with *Thaumatococcus danielli* fruit peel as the substrate
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

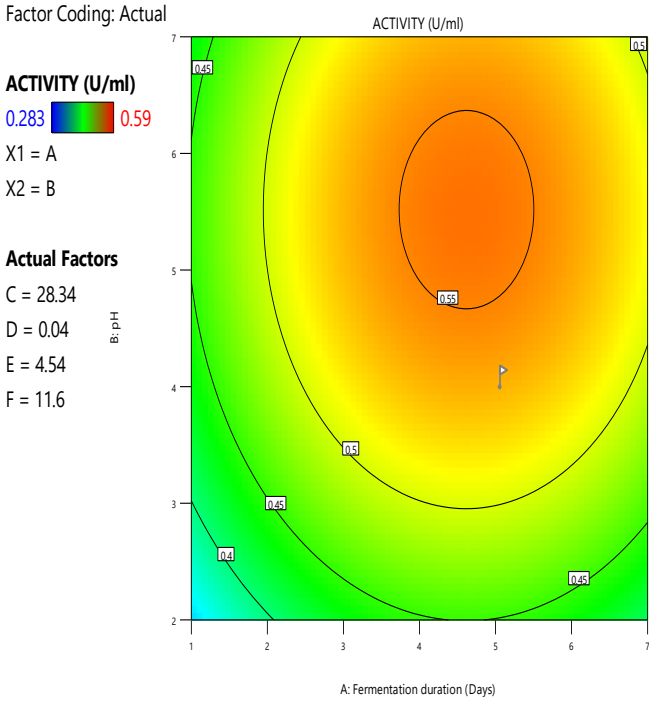

Figure 9: Effect of fermentation duration and pH on pectinase activity with *Pichia kudriavzevii* (strain F2-T429-5) with *Thaumatococcus danielli* fruit peels as the substrate
A - fermentation duration (d); **B** - pH; **C** - temperature (^{o}C); **D** - particle size (inch); **E** - inoculum volume (mL) and **F** - agitation (min)

The various optimum predicted fermentation conditions implied their respective suitable conditions for the production of the enzyme using the various fungi and substrate combinations. The differences in the predicted fermentation durations suggested the preferred period for each of the fungi to optimally utilize the substrate (pectin) in the agricultural wastes for the production of pectinase with optimum activity (Figures 1-9). Some fermentation durations have been reported to produce pectinases in literature. Six days[32] of fermentation has been reported for the production of pectinase with maximum activity, after which, the activity declined.[33] In another study, the pectinase activity peaked at day 4 and declined thereafter.[34] The difference in the optimum fermentation durations with the present study for *Aspergillus niger* could be due to the different strains of fungi used in their study (*Aspergillus fumigatus*).[34] The claim regarding the differences in the results for the predicted optimum conditions and pectinase activities have also been observed in the results for the three fungi employed for the production of pectinase in this study (Fig. 1-9). Additionally, the source of the microbe and the source plus the type of the substrates used could have also influenced the conditions obtained (in the study), leading to the differences in the predicted optimum activity of the pectin enzyme produced compared to literature (Figures 1 - 9). *T. danielli* fruit wastes' pectinase activity resulted in lower activity compared to *Citrus sinensis* peels for pectinase production, a similar lower effect was established when comparing the moulds to the isolated yeast strain used in the study (Table 2). However, despite the activity of the new substrate, *T. danielli* fruit waste' was shown to have lower activity for pectinase activity (Figures 7 - 9). The inclusion of the wastes in the optimization process (in this study) was due to the dearth of information in its use for pectinase production, with respect to the approach in this study, despite its relative abundance in parts of West Africa.[35-37]
The acidic conditions for which accounted for the optima production of the pectinases (Figures 1 to 9) were indications of acidic pectinases. The pH conditions and relatively mild temperature conditions for optima pectinase activities indicated the suitable environment for practical application of the enzyme, as in extraction of fruit juices.[10,16]

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These findings imply that, at higher conditions beyond the best pH and temperatures (Figures 1 to 9) for the enzyme production, the activities decreased. The reduction in activities could have been due to the denaturation of the secreted enzyme since the pectin enzyme is a protein.[10] Additionally, high conditions for both temperature and pH during the fermentation process would have led to an unfavorable environment for the microbes leading to fewer secretions of the enzyme to utilize the substrates, resulting in lower pectinase activities. For microbes to effectively act on the substrate, the surface area of such substrate needs to be increased; hence, the need for optimization of the surface area in the study via the inclusion of particle size for each substrate in the conditions for optimization. Jacob and Prema,[38] support this notion. Thus, optimum substrate particle size for the microbe to initiate the corresponding enzyme release is vital, resulting in the optimum activity of the enzyme produced. Also, the microbial volume was optimized because competition for substrates with a high concentration of microbes relative to the amount of substrates could result in reduced enzyme activity.[33,39,40]

Since the medium for fermentation utilized was solid-state, the secreted enzymes would have adhered to the fermented medium hence, the need for agitation to extract the enzyme into the extraction solvent. Above the optimum duration of agitation for best activity, this action would have led to the modification of the enzyme's active sites (due to agitation being a mechanical process), thereby reducing the catalysis of the enzyme's active sites with the pectin (the substrate).[10] It is worthy of note that the factors considered: fermentation duration (A), pH (B), temperature (C), particle size (D), inoculum volume (E), and agitation time (F) largely had significant effects ($p < 0.05$) in improving the activity of the pectin enzyme secreted by the various fungi, with few exceptions (Table 3). The exceptions were indications of the non-significance ($p > 0.05$) of each of the stated conditions' influence on the optimum pectinase activities obtained (Table 3). Hence, the Box-Behnken design can be used to show the parameters/conditions with positive and/or negative effects on the pectinase activity produced (in real-time), as confirmed in the study of Mohandas *et al.* (2018).[11]

Table 3: p-values for the Quadratic model (ANOVA)

Variables	**FIGURES**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Model	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0109	< 0.0001	< 0.0001	< 0.0001
A-Fermentation duration	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
B-pH	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0225	< 0.0001	< 0.0001	< 0.0001
C-Temperature	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.4494	< 0.0001	< 0.0001	< 0.0001
D-Particle size	< 0.0001	< 0.0001	0.0691	< 0.0001	< 0.0001	0.8225	< 0.0001	< 0.0001	0.1649
E-Inoculum volume	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1319	< 0.0001	< 0.0001	< 0.0001
F-Agitation during extraction	0.0005	0.0528	0.0043	< 0.0001	< 0.0001	0.7356	0.0015	< 0.0001	0.0844
AB	0.9739	0.8564	0.9691	0.7839	0.0117	0.8086	0.5796	0.5669	0.9861
AC	1.0000	0.7280	1.0000	0.8909	0.9953	0.2593	0.1235	0.2475	0.0794
AD	0.9816	0.8826	1.0000	0.0608	0.0656	0.5859	0.2377	0.6559	0.5008
AE	1.0000	1.0000	1.0000	0.0193	0.9953	0.5611	0.2215	0.8201	0.8894
AF	0.9479	0.7280	0.9691	0.8909	0.9953	0.2773	0.9177	0.1504	0.9169
BC	0.9479	0.0172	0.9846	0.7839	0.9953	0.5020	0.0103	0.9955	0.9031
BD	0.9739	0.9889	1.0000	0.7839	0.0114	0.8761	0.8464	0.6538	0.6395
BE	0.9632	0.0819	1.0000	0.6983	0.9900	0.4155	0.0778	0.8470	0.3181
BF	1.0000	0.9889	0.9846	1.0000	1.0000	0.7300	0.3368	0.7204	0.7414
CD	0.7198	0.9778	0.0063	0.8909	0.9906	0.7993	0.0167	0.9682	0.9446
CE	0.9479	0.0172	0.9846	0.7839	0.9953	0.9560	0.3695	0.7460	0.3975
CF	0.7996	0.8056	0.3559	0.8462	0.9900	0.2615	0.2207	0.0060	0.7495
DE	1.0000	1.0000	1.0000	0.0193	0.9953	0.7754	0.1235	0.2803	0.8894
DF	0.7690	0.9778	0.0190	0.8909	1.0000	0.8403	0.6987	0.3696	0.2433
EF	1.0000	0.9889	0.9846	1.0000	0.9906	0.4565	0.6517	0.1888	0.5909
A^2	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1070	< 0.0001	< 0.0001	< 0.0001
B^2	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.2815	< 0.0001	< 0.0001	< 0.0001
C^2	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0060	< 0.0001	0.0001	< 0.0001
D^2	< 0.0001	< 0.0001	0.0083	< 0.0001	0.0041	0.0057	< 0.0001	0.0001	0.0003
E^2	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1468	< 0.0001	0.0087	< 0.0001
F^2	0.0056	0.0002	0.0003	< 0.0001	< 0.0001	0.0457	< 0.0001	< 0.0001	0.0003

Note: p-values less than 0.0500 indicate the model terms were significant A - fermentation duration (d); B - pH; C - temperature (^oC); D - particle size (inch); E - inoculum volume (mL) and F - agitation (min). 1, 2, 3, 4, 5, 6, 7, 8, and 9 represent the p-values for Figures 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively.

Conclusion

The fungi and substrates utilized in the study can be locally employed in pectinase production. However, the best substrate and microorganism established in the study for pectinase production with optimum pectinase activity was the *Citrus sinensis* (orange) peel and *Aspergillus niger*, respectively. Optimization of pectinase activity with *Pichia kudriavzevii* (strain F2 T429-5) and *Thaumatococcus. danielli* fruit wastes also showed the production of the pectinase. The study, therefore, has introduced an alternative substrate and microorganism for pectinase production. Subsequent studies can target the improvement of pectinase production using the findings from this study, through genetic engineering to further improve the novel yeast strain used in the study. Extraction of the pectin (substrate) from the plant for enhancement in enzyme production should be studied. Additionally, a higher-order or a more suitable model to optimize *P. kudriavzevii* (strain F2-T429-5) in *Triticum aestivum* bran fermentation for better prediction of the pectinase activity be investigated. Furthermore, our laboratory is exploring the possible utilization of other models, for the independent analysis of *P. kudriavzevii* (strain F2-T429-5) in *Citrus sinensis* peel, and in *Thaumatococcus danielli* fruit wastes; plus, *Penicillium sp* in *Triticum aestivum* bran and *Thaumatococcus danielli* fruit wastes, with a view to enhancing model fitness.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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