Optimization of Pectinase Activity From Locally Isolated Fungi and Agrowastes

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Abstract

Background: Pectin enzymes are biocatalysts that degrade pectin into simpler forms. Fermentation is the commonly utilized method for pectinase production. Prior to optimization of pectinase activity, preliminary findings were undertaken to select the best screened microbe (*Aspergillus niger*), agrowastes and extraction solvents. Solid-state fermentation was employed in the study (optimization process), utilizing the Box-Behnken design in Design-Expert software package version 12.0.3.

Results: The results showed 0.1 molar sodium chloride as the best extraction solvent, with the activity higher than the citrate buffer. However, pectinase activity obtained with distilled water was significantly ($p<0.05$) lower than 0.1 molar sodium chloride. For the substrates employed in the study, the citrus (orange) peel had the highest pectinase activity of ≈0.40 mg per ml. The activity of citrus peels was significantly higher than the activities from each of corn cob, banana peel, wheat bran, *Thaumatococcus danielli* fruit wastes and *Thaumatococcus danielli* leaves. A significant increase ($p<0.05$) in pectinase activity was also obtained with *Thaumatococcus danielli* fruit wastes relative to *Thaumatococcus danielli* leaves. *Pichia kudriavzevii* strain F2-T429-5 and *Pichia kudriavzevii* strain CY902 have been identified to complement for pectinase production. From the results obtained, the optimum conditions for pectinase production were approximately 5.87 days of fermentation; pH of 3.90; at 21.24 ^{o}C; particle size of 0.06-inch; inoculum volume of 1.00 ml, and agitation time (for pectinase extraction) at 11.43 minutes. Optimisation of the selected conditions using the Box-Behnken design and analysis as a statistical tool resulted in a higher activity.

Conclusion: Local production of pectinase would help in reducing hunger through local job creation, thereby positively contributing to the Sustainable Development Goals (SDGs).

Background

Pectinase is an enzyme that depolymerise/split polygalactouronic acid to monomers through glycosidic and esterification cleavages [1-3].The food processing industries like brewery, fruit juice industry etc. are currently the largest user of food enzymes with market growth of USD 559.2 million in 2019 [4]. In addition, pectinases make up almost 25 % of the global food enzyme market [5]. Pectinase finds application in fruit juice industries such as in the extraction and clarification of these juices; thereby enhancing the yield of the resultant product (the juice) [2,5]. Interestingly, pectinase reduces production costs leading to higher yield, less equipment required, and less labor, especially during juice extraction and concentration [2,6-8]. Pectinases could also be employed in the pulp and paper industry [9], textile industry [7], management of waste water [10] among many other areas that demand the breakdown of pectin materials. Unfortunately, despite the advantages offered by this enzyme and the high demand for the pectin enzyme by various industries in Nigeria, importation is still significantly embraced as the means to meet industrial needs.

Of the preferred source of micro-organisms used in production of pectinase [5,11,12], *Aspergillus niger* has attracted most attention because of its versatility and status as an organism with GRAS (Generally Regarded As Safe) status [12].

Also, the rising human population and urbanization are significant drivers that encourage the generation of wastes with implication on human health and the ecosystem, therefore a call for concern as regards its management in such a way that has less impact on human health and the environment at large has been expressed with converting wastes to wealth, thereby, providing a good use for the abundance of agrowastes [13-16].

Different studies have reported the use of agricultural wastes such as orange peels, mango peels, saw dust, wheat bran among many other substrates [5,13-15] as substrates for the enzyme production.

Nigeria, being one of the largest producers of citrus in Africa with obvious evidence of high rate of consumption, generation of abundant orange wastes in Nigeria is inevitable, which implies the need to effectively manage the wastes generation.

Thaumatococcus daniellii (Benn.) Benth, is a rhizomatous plant found in the tropical rain forests and coastal areas of West Africa, particularly, Nigeria, Ghana and Cote d'Ivoire [17,18]. It is also called 'sweet prayers' plant, miraculous fruit, miraculous berry, serendipity berry and katamfe/katempfe [17]. Whether cultivated or in the wild, contributes to the economy of the rural people in most parts of Southern Nigeria through its stalks, leaves, fruits and rhizomes [17,19,20]. Presently, *Thaumatococcus danielli* wastes in form of fleshly part generated after extraction of thaumatin, a group of intensely sweet proteins [17,21] by processing plants requires appropriate waste management, while the waste in form of its leaves discarded indiscriminately after use by the consumers for wrapping of local delicacies, is a call for concern. The good news is that, from these agrowastes in the environment, pectin enzymes could be produced from them. As literatures support the distribution of pectinases in higher plants and microorganisms (in the course of fermentation) in our immediate environment [22,23].

An appropriate and suitable medium under optimized conditions for production of pectinase is important because of its significant influence on the enzyme yield and activity. However, studies have reported the production of pectinase is influenced by a variety of factors such as pH, inoculum size, the particle size of the substrate(s), temperature, and fermentation duration; are recognized from studies to have significant impact on pectinase production [2].

One-factor-at-a-time (OFAT) optimization strategy is a tedious approach which could be either supplemented or replaced with statistical optimization techniques; as in the response surface methodology (RSM). The statistical design has been shown to provide efficient and more satisfactory approach than the OFAT optimization strategy; because, it can study many variables simultaneously with a lower number of observations, thus saving time and money/cost [24].

It is believed that subjecting agrowastes to solid state fermentation under appropriate conditions will not only improve the activity of enzyme of interest but help manage the implications associated with such waste. Hence, this study aimed at screening pectinolytic fungi from the environment, agricultural wastes and optimise selected conditions from the preliminary study and information from earlier study [2] for pectinase activity using the Box-Behnken Design, a statistical design.

Preliminary study

Majority of the factors optimized in the study were used earlier; using the OFAT method for optimization of pectinase using *Saccharomyces cerevisiae* (ATCC 52712) and application of the enzyme in juice extraction [2]. Some of the factors used in the earlier study and selected for use in the present study were; fermentation duration, extraction solvent, pH, temperature and agitation during extraction of the enzyme from the fermented substrate [2]. In addition, particle size of the substrate was also included in this study; as particle size influences microbe action on the substrate to releasing the enzyme, as shown in section for optimization of the enzyme activity.

Materials And Methods For Preliminary Study

Sample collection

The substrates used for solid-state fermentation were corn cobs, banana peels, wheat bran, orange peels, *Thaumatococcus danielli* fruit wastes, and *Thaumatococcus danielli* leaves. The *Thaumatococcus danielli* fruit wastes and *Thaumatococcus danielli* leaves were sourced from Owode-Yewa wild plant area (Atan-Ota) Ogun State, Nigeria. The banana peels were obtained from Canaan Land farms, Ota; Ogun State, Nigeria; while the other (fruit) substrates were purchased from the bulk fruit section in the Nigerian Army Shopping Arena, Oshodi, Lagos State; Nigeria. The substrates were then washed and dried in an oven for 48 hours at 58 ^{o}C before milling into a particle size of 0.097 inch in a Hammer mill to allow for an increase in surface area for microbial action.

Fungi isolation and screening

Deteriorated fruits were sourced from dumpsites and soils in the Federal Institute of Industrial Research, Oshodi (FIIRO), Lagos State, Nigeria. The samples were ground and serial dilution done before Pour-plating for microbial isolation, with streptomycin antibiotic added into the sterilized potato dextrose agar (PDA) media (to exclude bacteria growth); after which, each colony was then subjected to pure culture by streaking. Identification of the resultant pure culture and microscopically identified using some unique features [25]. After which, molecular identification of each isolated fungus was undertaken [26,27].

The screening was undertaken using the Czapek medium (Khattab et al., 2016). KNO_3, KH_2PO_4, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$, pectin, peptone, agar, and distilled water were used to prepare the media and sterilized. Microbes so obtained were each inoculated into the labeled plates and incubated at room temperature (25 ^{o}C ±2) for forty-eight hours. With potassium iodide solution added to enhance clarity for measurement of zone of clearance/hydrolysis. The best microbe (having the largest hydrolysis zone) was identified and used to produce pectinase.

Pectinase production

A solid-state fermentation process using each of the substrates with the best screened fungal isolate was then undertaken. For each microbe, 2 grams of each substrate was weighed into each labeled flask, and

addition of 2 ml of a nutrient medium composed of dilution of KCl, KH_2PO_4, KNO_3, K_2HPO_4, KNO_3, and $MgSO_4$ with adjustment of pH using 1M NaOH and HCl. Each substrate and 2 ml of nutrient solution combination in the conical flasks were then autoclaved at 121 ^oC for 15 minutes. After that, the flasks were cooled to room temperature, and 0.02 g (1 %) of citrus pectin as an inducer was aseptically added to the contents. Thereafter, inoculating with the best-screened microorganism (*Aspergillus niger* in Table 1) and incubated at 30 ^oC for six days [2,26].

Pectinase extraction

Prior to extraction of the enzymes in the six substrates, viz; corn cobs, banana peels, wheat bran, orange peels, *Thaumatococcus danielli* fruit wastes, and *Thaumatococcus danielli* leaves employed in the fermentation process, the phase involving crude pectinase extraction determination was undertaken using three extraction solvents viz; citrate buffer (pH 5.0), 0.1 M NaCl solution and distilled water with orange peel as the only substrate for fermentation. After which, the best extraction solvent obtained was then used for the extraction of all the substrates in Fig. 2.

Assay for pectinase activity

The procedure by Miller [29] was employed with modifications, as 3 ml of 1 % pectin solution prepared by dilution in distilled water and homogenized. One ml of the crude pectin enzyme was added, and the mixture was incubated at 50 ^oC in a water bath for 10 minutes. After which, 1 ml of freshly prepared Dinitrosalycyclic acid (DNSA) solution was added and boiled for 5 minutes and after that left to cool to room temperature on the laboratory bench. UV-Vis spectrophotometer was preset at 540 nm, with readings taken as absorbance and galacturonic acid standard curve used for pectinase activity determination.

Plotting the bar charts, analysis of variance (ANOVA), and t-test (for comparison) were undertaken using Microsoft Excel version 2016.

OPTIMIZATION OF THE PECTINASE USING THE OPTIMAL PARAMETERS IN (A).

The results from the preliminary section earlier undertaken as shown in Table 1 and Figures 2 and 3 informed the selection of the best fungus (*Aspergillus niger*), extraction solvent (0.1 M NaCl) and agrowastes (orange peel) for optimization.

Pectinase production

The same approach as regards production of pectinase, extraction and assay were adopted as mention above in the preliminary study. The only difference for this phase, was in the use of the already established optimal conditions obtained in the study for production in bulk.

Screening of variables by Box Behnken design

The quadratic model in Box-Behnken in the Design software package file version 12.0.3.0 was employed in Response Surface Design of the experiment and analysis of data obtained using the same software

package. The earlier study informed the selection of the ranges considered [2].

Six variables conditions were screened consisting of: fermentation duration (days), 1 to 7 days; pH, 2 to 7; temperature, 20 to 50 ^{o}C; particle size of orange peels, 0.027 to 0.097 inch; inoculum volume, 1 to 5 ml and agitation of fermented substrate prior to filtration of 10 to 60 minutes. Each factor of this experimental design was examined at three levels as shown by Table 1.

TABLE 1. **Experimental variables at 3 different levels used for the screening six variables for optimization of pectinase activity using the Box-Behnken design**

TABLE 1. **Experimental variables at 3 different levels used for the screening six variables for optimization of pectinase activity using the Box-Behnken design**

	A: Fermentation Day(s)	B: pH	C: Temperature °C	D: Particle size (inch)	E: Inoculum volume (ml)	F: Agitation (minutes)	Activity (mg/ml) Observed	Activity Predicted
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

Results

Preliminary results and discussion

Table 2 shows the isolated fungi screened for their pectinolytic ability, with the extent for pectinase production denoted by their zones of hydrolysis. From the solvents used for extraction, though citrate buffer (pH 5.0) and distilled water also were effective in extracting the pectin enzyme, 0.1 M NaCl resulted in the highest activity regarding extraction of the pectin enzyme (Fig. 1). Comparing each of the extraction buffers to 0.1 M NaCl, it was seen that there was no significant pectin enzyme activity ($p>0.05$) between citrate buffer (pH 5.0) and 0.1 M NaCl. However, 0.1 M NaCl had a significant increase ($p<0.05$) in pectinase activity when compared to distilled water (Fig. 1). Since the carbohydrate (pectin) broken down by the enzymes (a protein) secreted from the microbes were found to adhere to each other during the fermentation, the bonds existing between the secreted enzymes and carbohydrates in the fermented substrates needed to be broken for enzyme release in the filtrate; bonds such as hydrogen bonds, ionic bond and Vander Waal's forces are known to exist in the fermented substrates and the entrapped enzymes [30,31].

TABLE 2. Screening of the fungi

Isolates	Source	Pectin hydrolysis	Average Zone of clearance (mm)
Aspergillus niger (mold)	Agrowastes	+	83.5
Penicillium sp. (mold)	Agrowastes	+	72
Pichia kudriavzevii strain F2-T429-5 (yeast)	Agrowastes	+	37
(*Pichia kudriavzevii strain CY902)* (yeast)	Agrowastes	+	34
(*Saccharomyces cerevisiae)* (yeast)	Agrowastes	+	31

So, 0.1 M NaCl was most effective in extracting the pectin enzymes from the fermented substrates (Fig. 1). The extraction solvent (with the best enzyme extraction ability), 0.1.M NaCl, could be due to its ability to exhibit the most effect in salting-in relative to the other solvents studied, thereby enhancing the enzyme's extraction from the fermented medium [2, 32].

For pectinase activity using the different substrates (Fig. 2), there was also significant effect ($p<0.05$) between groups. In comparing each of the substrates to orange peel, a significant increase ($p<0.05$) in activity was obtained; signifying that, probably, orange peels as substrate for pectinase production had higher concentration of pectin which triggered more of the microbial pectin enzyme secretion in the medium as compared to the other substrates investigated. Comparison between *Thaumatococcus danielli* fruit wastes and its leaves (Fig. 2), the result showed that though they both produced pectin enzymes, the activity of the enzyme produced was each significantly lower than the orange peel. However, a comparison between

the *Thaumatococcus danielli* fruit wastes and leaves, a significant increase ($p<0.05$) in pectinase activity in the utilization of the fruit wastes was obtained. Suggesting that, with the very little-known use of the fruit wastes (relative its leaves), which are usually discarded and reported to account for about 95.2% of the fruit after harvesting the aril which has the thaumatin (sweetener). These fruit wastes can now be harnessed for pectinase production, as there is a dearth of literature with regards to its use in pectinase production [17].

Since orange peel was also used in determining the potency (activity) of 0.1 M NaCl as the best extraction solvent (Fig. 1), therefore, there is basis for comparison of the pectinase activities of the best extraction solvent in Fig. 1 and that of orange peel in Fig. 2 (for which 0.1 M NaCl was also used for extraction).

This comparison showed insignificance ($p>0.05$). The insignificant result suggests consistency as both experiments were performed using the same condition with respect to substrate and extraction solvent, but the only difference was that, Fig. 1 and Fig. 2 experiments were done at different times.

Results and discussion for optimization of the pectinase using the optimal parameters

The use of *Aspergillus niger,* orange peel and 0.1 M NaCl as extraction solvent, and following the design in Table 1 for the Box-Behnken design resulted in the results in Figure 3 to 7.

Fermentation duration (Fig. 3) was obtained to be optimum on 5 days 19.2 hours (approximately on the 6^{th} day) which signified that at this point, the microorganism (*Aspergillus niger)* was able to adequately split the pectin components in the substrates into simpler sugars with the release of corresponding pectinases in the medium; thereby resulting in higher activity. It therefore implies that, decrease in activity beyond this point could be attributed to catabolite repression and decline in the medium's pectin content [33,34]. Pectinase was produced at approximately 5.4 days (129 hours) with *Aspergillus fumigatus* [22]. The difference in the fermentation duration (in relation to the present work) could be due to the difference in the strain of microorganism, type and nature of substrates used.

The optimum pH (Fig. 4) of 3.9 (approximately 4.0) and temperature (Fig. 4) of about $21^{o}C$ could be due to the ability of the microorganism to produce pectinase in acidic pH and the fact that, the organism favored pectinase production at relatively mild conditions of temperature. This therefore signified that, the condition beyond the optimum could have resulted in the unfavorable growth of the microorganisms and possibly denaturation of the pectin enzymes produced since enzymes are sensitive to pH and temperature; thereby, causing alteration to the active sites of the enzymes and resulting in lower activity of the pectinase produced in the medium [5,35]. The insignificant effect ($p>0.05$) in the interaction between temperature and pH each with respect to duration of pectinase extraction could be due the fact that, while temperature and pH interacted during the fermentation process, extraction was done after fermentation. Though researchers have reported higher duration of fermentation in comparison to that obtained in this study, the relative difference could be due to the difference in the microorganisms and substrates utilized. Furthermore, the type of fermentation vessel or flasks used for agitation as well as the amount of substrates used in the fermentation process (as used in the present study) could have favored the surface area of the fermented substrates interaction with the extraction solvent, thereby leading to more shear stress at higher agitation beyond approximately 11 minutes (Fig. 5), resulting in reduction in the enzyme's activity [36].

The optimum particle size of 0.06-inch (Fig. 6 and 7) in 1 ml of the inoculum (Fig. 6) (of *Aspergillus niger*) could have resulted in adequate increase in surface area of the substrates to the microorganism's action and more effective in favoring the microbial growth condition with little saturation of the fermentation media to about 5.9 days for best activity.

The coded factors for pectinase activity prediction of response for the factors is summarily indicated in the equation below:

ACTIVITY= +0.5325 +0.0211*A +0.0352*B -0.0120*C +0.0115*D +0.0138*E -0.0009*F -0.0000*AB +0.0000*AC +6.250 -06*AD +0.0000*AE -0.0000*AF +0.0000*BC -0.0000*BD -0.0000 BE +0.0000 BF -0.0001CD -0.0000 CE +0.0001CF +0.0000 DE -0.0001 DF +0.0000 EF -0.0207*A^2 -0.0352*B^2 -0.0113*C^2 -0.0135*D^2 -0.0131*E^2 -0.0010*F^2

Where A is fermentation duration (days); B- pH; C-Temperature; D- particle size; E- Inoculum size and F is agitation during extraction (in minutes).

Conclusion

The dearth of literature as regards pectinase production using *Thaumatococcus danielli* fruit wastes and its leaves, plus the use of new yeasts strains (*Pichia kudriavzevii* strain F2-T429-5 and *Pichia kudriavzevii strain CY902*) have been identified to complement for local pectinase production. Adopting this, would lead to a more profitable utilization of the fruit wastes which are presently underutilized. The confirmation of 0.1 M NaCl as extraction solvent is a good indicator, since acetate buffer, citrate buffer and distilled water were the predominantly known extraction solvents used in pectinase extraction. This study further confirms the feasibility in the use of locally sourced *Aspergillus niger* and citrus (orange) peel substrates in optimizing pectinase production. This study will encourage industrial scale up of pectinase production (from the equation for prediction of the coded factors), and boost sectors in need of this enzyme in the enhancement of their output. Thereby, hopefully aid in addressing some of the concerns in the millennium development goals such as, poverty reduction through job creation (by encouraging the local production of pectinase), sustainable and efficient utilization of natural resources as well as promotion of industrialization.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the study can be made available from the corresponding author on reasonable request.

Conflict of interest

The authors declare no conflict of interest.

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Author's contributions

Idea conceived and executed by George D. Ametefe, sourcing of some substrates with preparation for fermentation by**Lemo A. Oluwadamilare, preparation of media for fungal growth by Ifeoma C. James, and Olubunmi I. Ibidapo took the absorbance readings in spectrophotometer. Preparation of screening media by Vera O. Ofoegbu, interpretation of spectrophotometer readings by Folake Fashola. Laboratory supervision (on-site) by Frank A. Orji; with Emeka E.J. Iweala and Shalom N. Chinedu as the corresponding author's co and main supervisors respectively. As this study is part of George D. Ametefe's school dissertation.**

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Significance statement

This study investigated the relative potential in the use of isolated fungi, some agrowastes and selected extraction solvents for the improvement in the activity of pectinase. This study investigated the inherent benefit in the optimization of pectinase activity during production with the Box-Behnken Design using orange peels as substrates. Thereby, further maximizing the activity of pectinase for efficient application.

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Figures

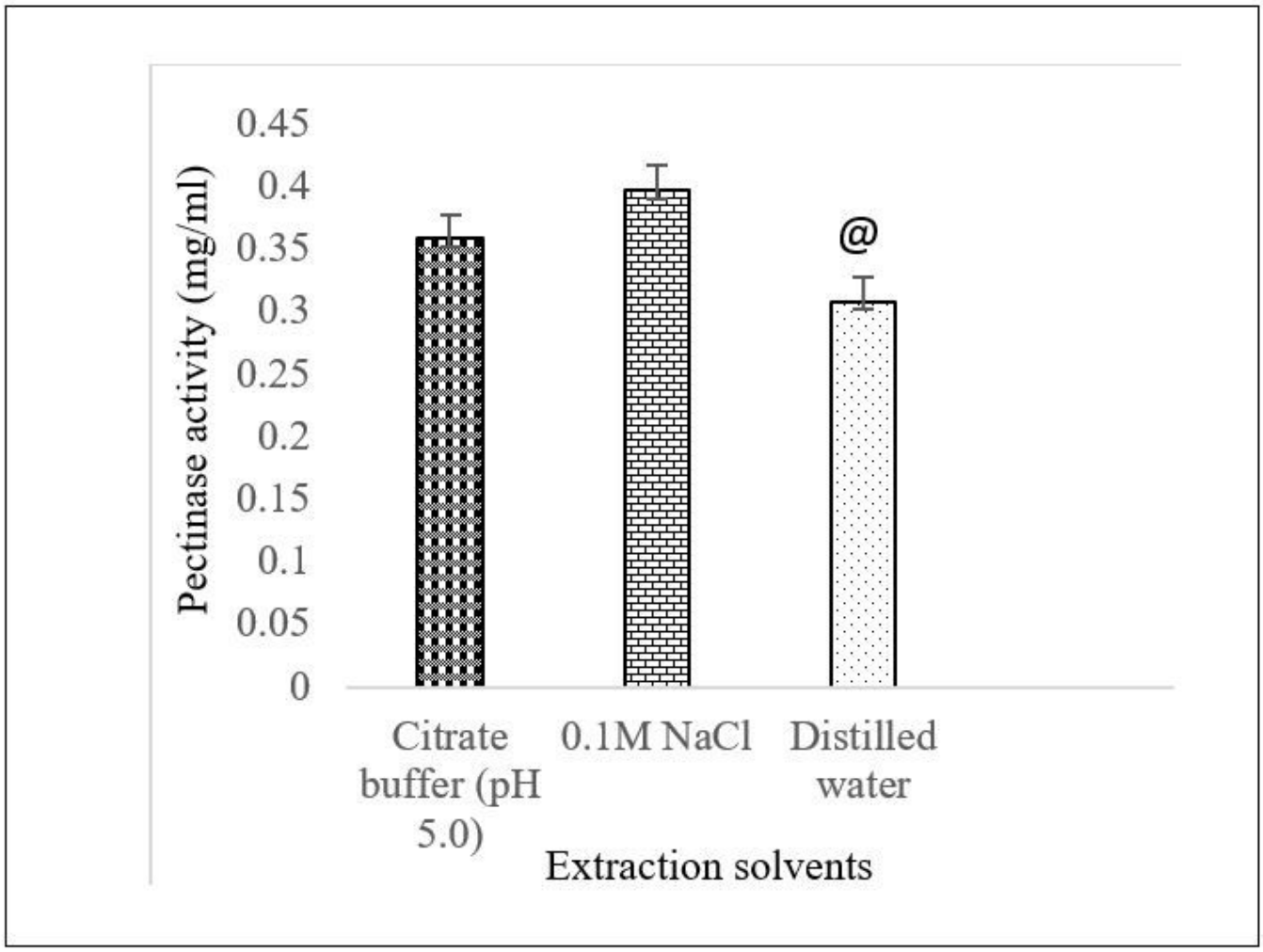

Figure 1

Effect of extraction solvents on pectinase activity. Where @ in Figure 1 signifies a significant decrease ($p<0.05$) between 0.1 M NaCl and distilled water.

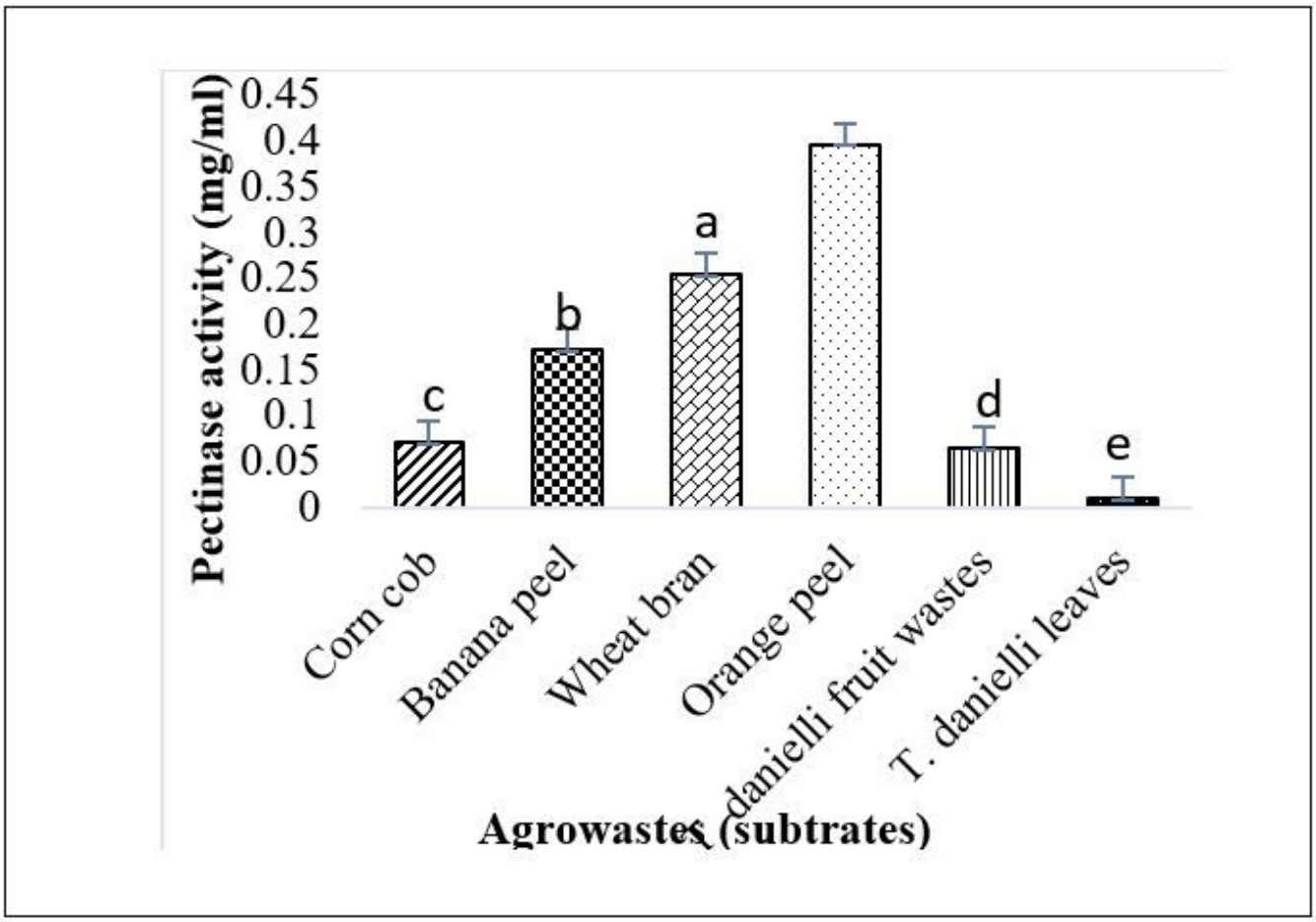

Figure 2

Effect of different agrowastes on pectinase activity. Where a, b, c, d and e signify significant decrease between each of the corresponding substrates and orange peel

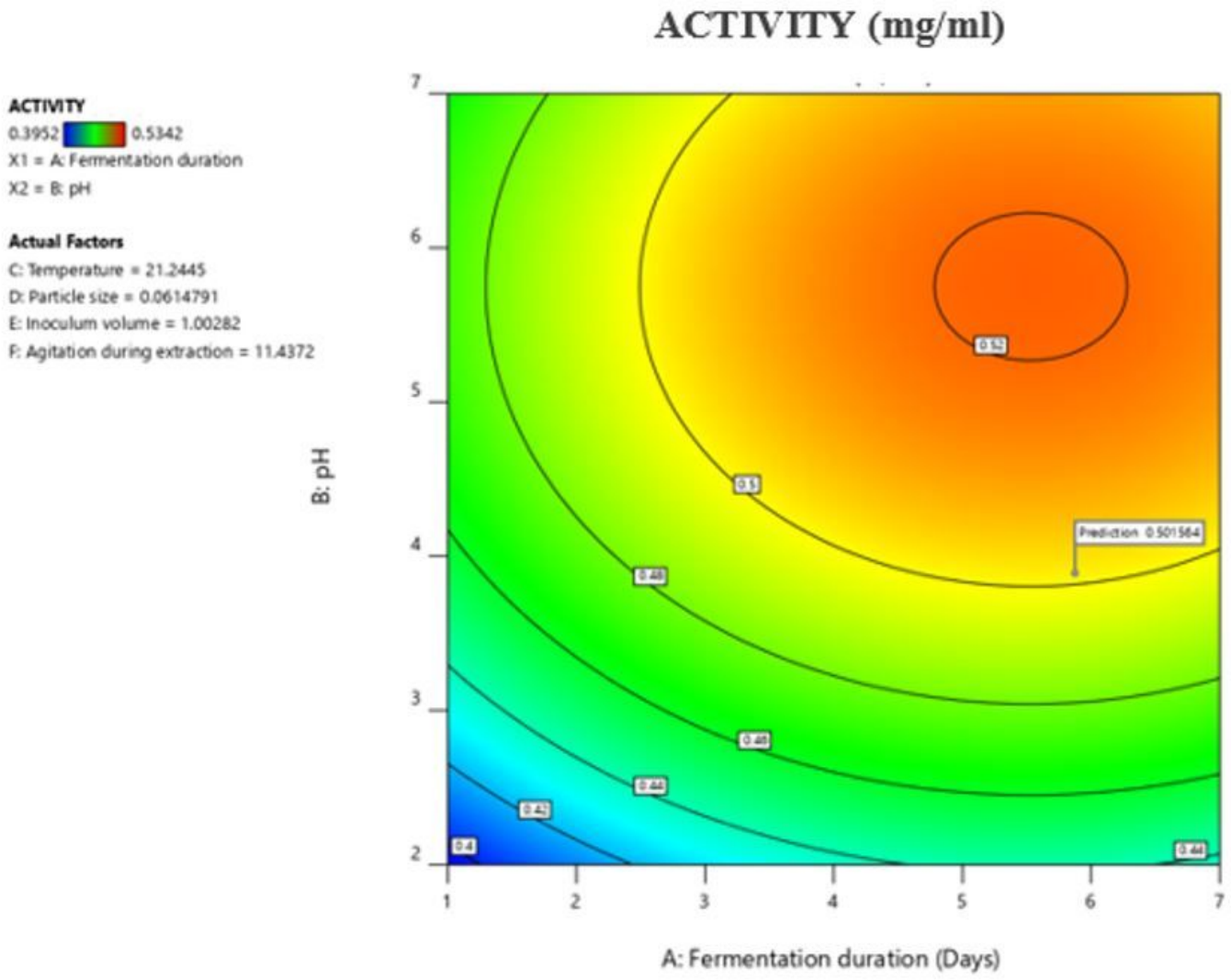

Figure 3

3D surface plot showing effect of fermentation and pH on pectinase activity

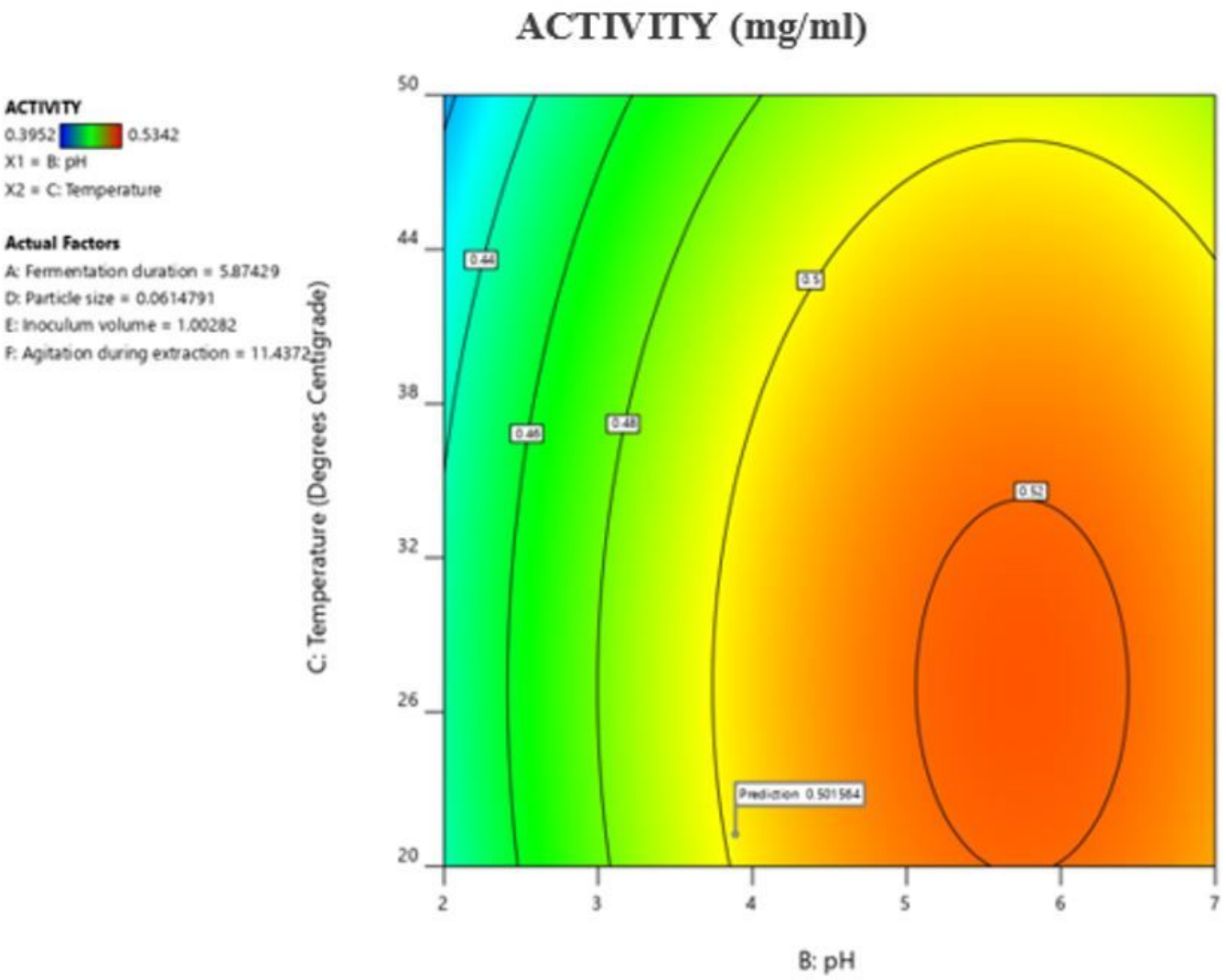

Figure 4

3D surface plot showing effect of pH and temperature on pectinase activity

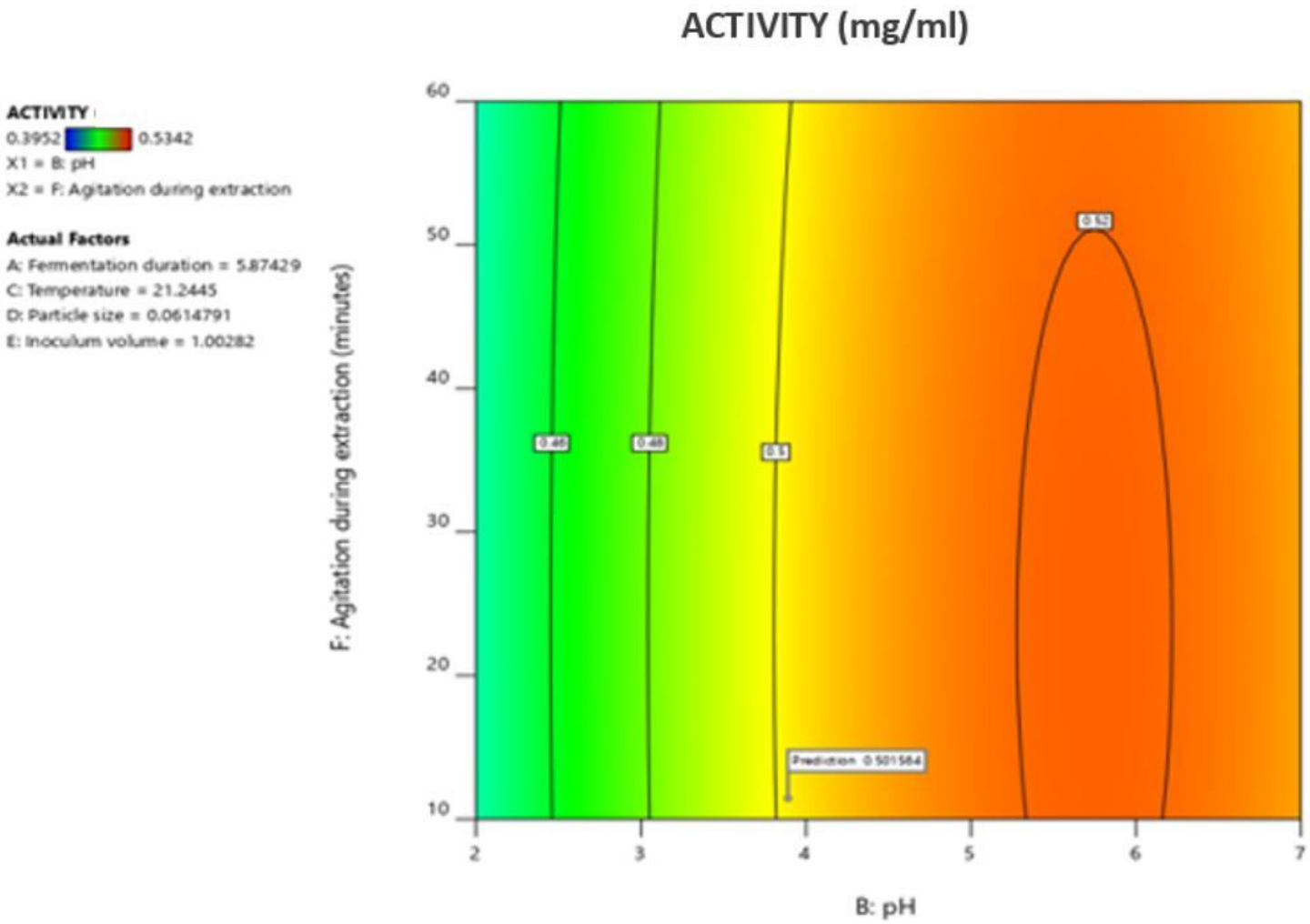

Figure 5

2D Contour plot showing effect of pH and agitation during pectinase extraction on pectinase activity

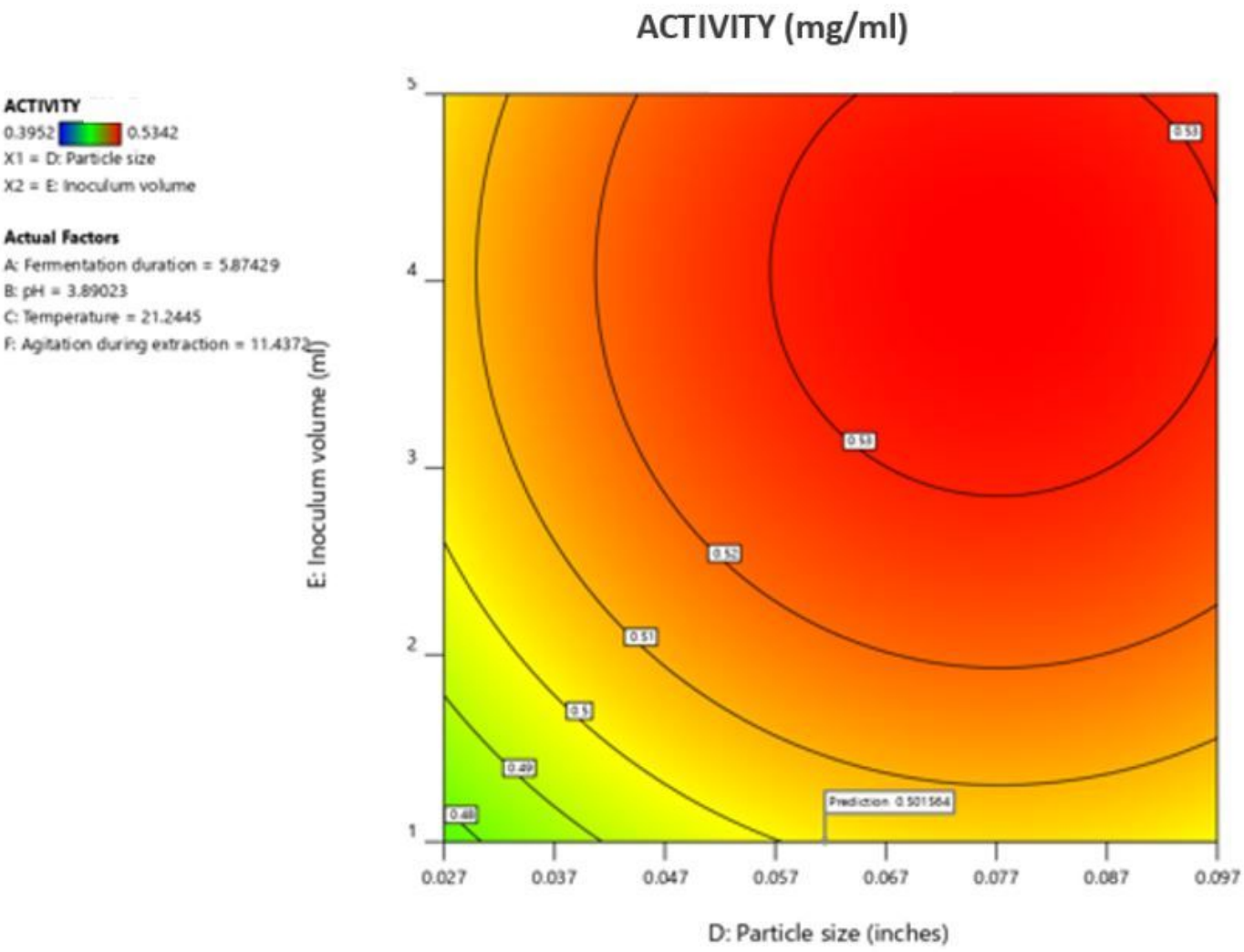

Figure 6

2D Contour plot showing effect of particle size and inoculum volume during pectinase-on-pectinase activity

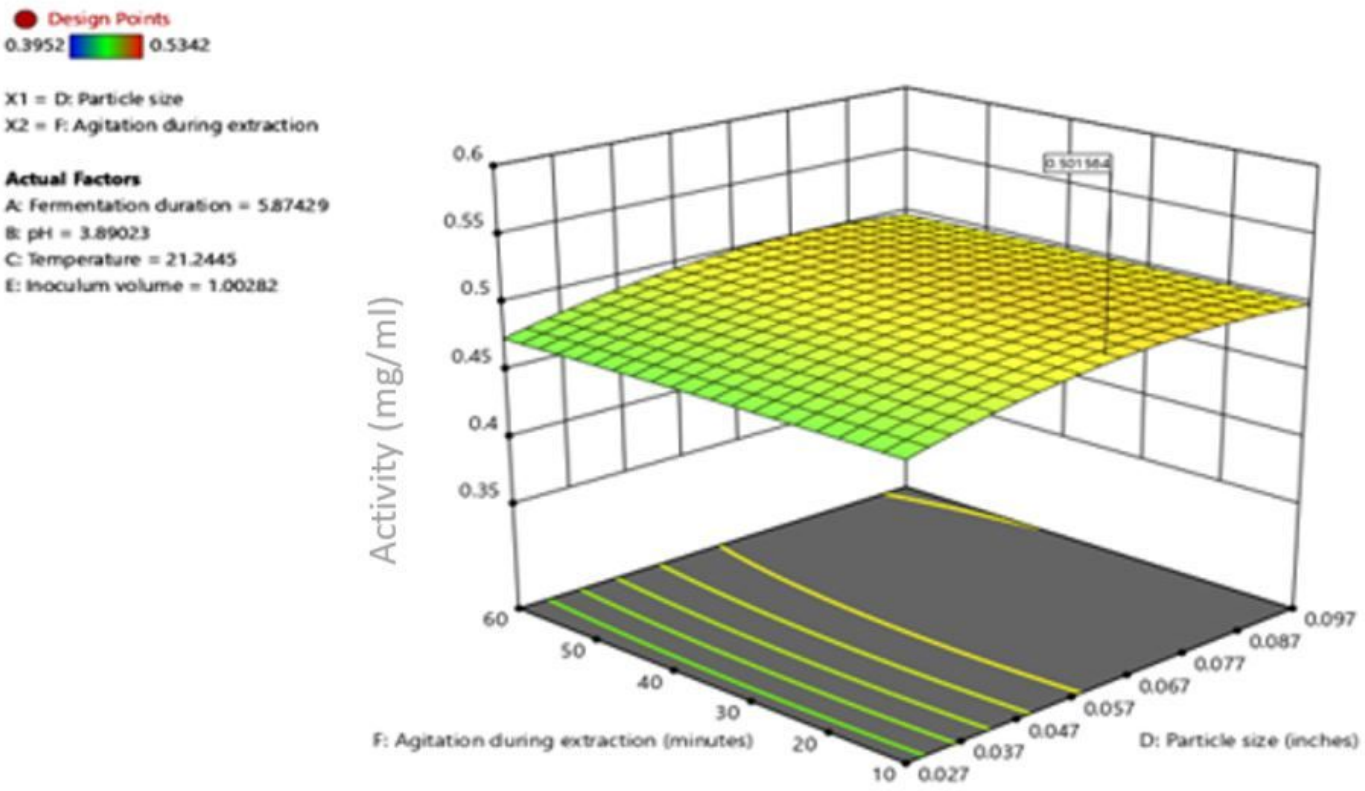

Figure 7

3D contour plot showing effect of particle size and agitation during extraction on pectinase activity