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Optimization of Endo-Pectinase and Pectin Lyase Production from Wheat Bran by *Bacillus pumilus* using Response Surface Methodology

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Highlights

- This paper focuses on pectin lyase and endo-pectinase synthesis from wheat bran by *Bacillus pumilus*.
- RSM was employed.
- Optimum conditions for enzyme production were obtained.

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Keywords

Endo-pectinase Optimization, Pectin lyase, Response surface methodology (RSM), Wheat bran

Abstract

Wheat bran is one of the most used agricultural residues for enzyme synthesis. It has a high organic content; thus, it causes environmental pollution. In this study, wheat bran was selected as a carbon source and it was supplemented with yeast extract and ammonium sulphate. Endo-pectinase and pectin lyase production by *Bacillus pumilus* was performed in a batch system. RSM was applied to examine the effects of the wheat bran, yeast extract and ammonium sulphate concentrations on the synthesis of enzymes and the dosages of these nutrients were optimized. According to the model design, the optimum conditions were 4.74% w/v wheat bran, 0.12% ammonium sulphate and 0.12% yeast extract. The high values of R^2 and R^2_{adj} indicated that the fitted model showed good agreement with the predicted and actual values. In conclusion, these studies revealed that wheat bran can be used for the production of endo-pectinase and pectin lyase enzymes based on high enzyme activities.

1. INTRODUCTION

Pectic substances are complex polysaccharides of higher plants and are found mainly in the middle lamella between cells in tissues [1]. Pectin, protopectin, pectinic acid and pectic acid are basic types of pectic substances. Pectinases are clusters of enzymes that break down pectic substances by various mechanisms. They are classified as protopectinases, esterases and depolymerases, according to the attack mechanism on the galacturonan part of the pectin molecule. Protopectinases are involved in the degradation of insoluble protopectin and provide the formation of highly polymerized soluble pectin. Esterases remove methoxy esters by catalyzing the de-esterification of pectin. Depolymerases are the enzymes that act on pectic substances by hydrolyzing the glycosidic linkages between galacturonic monomers or cleaving α-1,4-glycosidic linkages by trans-elimination [2-4]. Pectinolytic enzymes are among the first enzymes that were used in households. Their industrial usage began in 1930 with the production of fruit juice and wine [5]. Today, pectinolytic enzymes account for approximately 25% of the world's manufacturing of food enzymes. Pectinases have widespread usages in the textile, paper, food manufacturing, tea and coffee fermentation, olive oil industries and in the treatment of pectic wastewater [6,7]. They are used as juice yield and color enhancers, clarifies, and in fruit mash treatment in the fruit juice industry [8,9]. They are also used in the clarification of wine [10]. Pectinases are also classified as alkaline pectinases and acidic pectinases according to their usage area. Alkaline pectinase is synthesized efficiently by bacterial sources and are primarily utilized in the textile industries and for pectic wastewater pretreatment occurring from the fruit juice industry. Acidic pectinases produced by fungi are used in the fruit juice industry [4].

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Two methods of fermentation are used in the synthesis of these enzymes: submerged fermentation (SMF) and solid-state fermentation (SSF) [11]. In SMF, a liquid nutrient medium is used for the cultivation of the microorganisms. On the other hand, SSF includes the production of microorganisms on a solid substrate. The main concerns in enzyme production process are yield, stability, specificity and production cost. The composition of the nutrients is one of the key factors that influences microbial growth and enzyme synthesis [12]. Approximately 30-40 % of the enzyme production costs constitute the fermentation medium [13]. Castilho et. al [14] studied lipase fermentation by *Penicillium restrictum* using SSF and SMF techniques and reported that the investment cost required for SSF is 78 % lower than that required for SMF. Agricultural residues and food waste such as sugar beet pulp, wheat and corn flour, rice bran and wheat bran can be selected as the solid material for SSF [15,16]. This type of waste contains high amounts of carbohydrates and other nutrients. However, if they do not include all the nutrients required for the enzyme production processes, extra carbon or nitrogen sources can be added to improve the fermentation medium [17]. Using these wastes as carbon sources in the manufacturing of enzymes decreases manufacturing costs and helps to solve the problem of disposal. SSF also has other advantages over SMF. For example, the growth medium is very similar to the natural habitat of microorganisms, thus high volumetric productivity and high enzyme yields can be obtained. In addition, the amount of liquid waste produced with SSF is lower than SMF [14].

Wheat bran is an agricultural residue preferred by many researchers. In 2017, the Food and Agriculture Organization of the United Nations announced that the wheat production around the world was 771,700,000 tons. Approximately 15–20% (in weight) of wheat is removed as wheat bran. Therefore, in SSF processes, wheat bran is classified as a sustainable by-product. This complex carbohydrate can easily be utilized by microorganisms that obtain pectinases. The use of such an abundant material to obtain a value-added enzyme can also assist to overcome the pollution issue [12].

In fermentation processes, the effects of each parameter on the system can be examined one by one with the classical optimization methods. However, the optimization of media components using such methods has several drawbacks. For example, these methods require a lot of experimentation, which would take a lot of time. In addition, in multivariable systems, these methods are sometimes not sufficient to explain the interactions between the variables. Therefore, in recent years, classical methods have been replaced by statistical methods. One of the most commonly used statistical methods is the response surface methodology (RSM). It involves factorial designs and regression analysis to plan multifactor experiments and solve multi-variate equations [13,18]. It enables the planning of multifactor experiments, determination of optimum experimental conditions, constitution of empirical models (equations) and assessment of the importance of independent variables on the desired variable response [19]. It has been commonly used for optimization in the fields of biochemical and chemical engineering to produce enzymes, determine the composition of cultivation media, conditions of enzymatic hydrolysis and parameters for the polymers and food production [18,19]. In this study, wheat bran was selected as the carbon source and supplemented with ammonium sulphate $(NH_4)_2SO_4$ and yeast extract. RSM was employed to examine the effects of the wheat bran, yeast extract and ammonium sulphate concentrations on the synthesis of pectin lyase and endo-pectinase and the dosages of these nutrients were optimized.

2. MATERIAL METHOD

2.1. Bacterial Strain and Growth Conditions

The *Bacillus pumilus* (NRRL B-212), which was provided from USA (National Center for Agricultural Utilization Research) was grown on potato dextrose agar at 30 ^{o}C and then inoculated in the nutrient solution. The production of the microorganism was achieved in a sterilized (autoclaved at 1.1 atm and 121 ^{o}C) growth medium. The composition of this medium and the culture conditions were explained in the previous study [20]. After incubating, the microorganisms were transmitted into the pectinase fermentation medium (in a 1:10 ratio).

2.2. Production of Endo- Pectinase and Pectin Lyase with Agricultural Wastes

Wheat bran used in the SSF studies was obtained locally. Firstly, it was washed then dried in an oven at 60 ^{o}C for 24 h. The dried materials were then milled and sized (20-100 mesh). The solid material was placed Erlenmeyer flasks (250 mL) for enzyme production. The flasks were then sterilized for 15 minutes at a temperature of 121 $^{\circ}$C and cooled to room temperature. The tap water was used for hydrating the dried materials. After inoculation with liquid culture (10%, v/v), enzyme production experiments were conducted at 30 °C, pH 8 and 150 rpm. The enzyme activity measurements were performed for the samples centrifuged at 5000 rpm for 5 minutes.

2.3. Determination of Pectin Lyase and Endo-Pectinase Activities

The thiobarbituric acid method defined by Nedjma et al. [21] was used for the assay of pectin lyase activity. The method was explained in detail in our previous study [22].
Endo pectinase activity was measured viscosimetrically using a vibroviscometer (SV10, Sine Wave Vibro Viscometer, A&D Engineering) [23]. The reaction mixtures consisted of 1 mL of enzyme solution and 19 mL of 0.5 % (w/v) apple pectin in a 0.05 M glycine-NaOH buffer (pH 10). This solution was heated at 30 ^{o}C for 15 min. After incubation, the reduction in viscosity was monitored by a vibroviscometer. Under these conditions, the enzyme quantity reduced the initial solution viscosity by 50% per minute was determined as one unit of endo pectinase activity.

2.4. Optimization of Enzyme Production Process Parameters

In the present study, the relationship between the substrates and enzyme production was investigated using RSM. As a full factorial matrix, a central composite design (CCD) was applied to reveal the parameters influencing enzyme activity. According to a full factorial design, the value of α is equal to $(2k)^{1/4}$. In this study, the values of k and α were equal to 3 and 1.68179, respectively. Concentrations of wheat bran, $(NH_4)_2SO_4$ and yeast extract were used as numeric factors. To obtain a second order response surface, their quantities were changed at five different levels (-α, −1, 0, +1, +α) (Table 1). The maximum and minimum values of the substrate concentrations were selected according to the results of the initial tests. A total of 20 experiments were planned using this full factorial matrix. These experiments contained six center points, six axial points and eight factorial points with six replicates around center point (Table 2). Design Expert software Ver 7.0 (Stat Ease Inc.) statistical package was used to calculate and analyze of the second-order polynomial coefficients.

Table 1. *Levels of the parameters in CCD*

Independent variables (concentration, w/v %)	Symbol code	Range and levels				
		- 1.682(-α)	-1	0	+1	+1.682(+α)
Wheat bran	A	2.0	3.22	5.0	6.78	8.0
Ammonium sulphate	B	0.0	0.12	0.3	0.48	0.6
Yeast extract	C	0.0	0.12	0.3	0.48	0.6

3. RESULTS AND DISCUSSION

In this study, wheat bran was selected as the carbon source and supplemented with $(NH_4)_2SO_4$ and yeast extract. RSM was applied to examine the effects of the substrate concentrations on the synthesis of pectin lyase and endo-pectinase and dosages of these nutrients were optimized. The concentrations of the substrates were selected as the independent variables. Table 1 shows the levels and the range of these variables, while Table 2 presents the coded values of the factors, design and results of the experiments. The experimental findings showed that the pectin lyase and endo-pectinase activities matched to the second-order quadratic equation, providing two numerical correlations to predict the enzyme activity responses. The following model equations (Equations (1) and (2), in the coded factors) were proposed for the endo pectinase and pectin lyase activities, respectively:

Endo-pectinase activity = $+174.50 + 0.29\,A - 9.20\,B - 22.25\,C + 0.32AB - 3.56\,AC + 24.27\,BC - 34.07A^2 - 5.27\,B^2 - 24.49\,C^2$ (1)

Pectin lyase activity = $+26.26 - 2.15\,A - 4.74\,B - 10.72\,C - 2.16\,AB + 2.30\,AC + 4.21BC - 8.01A^2 + 0.26B^2 - 1.0\,C^2$. (2)

Table 2. *CCD matrix, Response factor results*

Runs	Variables			Responses			
				Endo-pectinase activity (U/mL) R1		Pectin lyase activity (U/mL) R2	
	A	B	C	Exp.	Predic.	Exp.	Predic.
1	-1	1	-1	90.61	95.27	29.67	25.90
2	+1.682	0	0	93.38	78.62	9.77	8.587E-004
3	0	0	0	167.31	174.50	23.90	26.26
4	1	1	1	85.44	100.55	6.20	4.25
5	0	+1.682	0	150.16	144.12	11.73	19.03
6	0	0	0	173.46	174.50	23.80	26.26
7	1	-1	-1	157.93	169.92	24.33	34.91
8	0	0	0	178.64	174.50	24.40	26.26
9	0	0	-1.682	144.98	142.65	49.07	41.47
10	1	-1	1	62.14	69.76	1.73	9.64
11	0	0	0	175.08	174.50	27.80	26.26
12	-1	1	1	106.15	106.44	14.73	8.29
13	1	1	-1	103.56	103.61	9.43	12.67
14	0	0	0	178.64	174.50	29.87	26.26
15	-1	-1	-1	165.70	162.87	33.40	39.49
16	-1.682	0	0	80.26	77.65	3.32	7.24
17	0	0	+1.682	82.85	67.81	3.65	5.40
18	-1	-1	1	64.72	76.95	4.13	5.02
19	0	-1.682	0	186.41	175.08	48.13	34.98
20	0	0	0	170.87	174.50	26.80	26.26

As can be seen from Equation (1), *A, AB* and *BC* had positive influences on the endo pectinase activity, whereas the other parameters had adverse effects. Similarly, *AC*, *BC* and B^2 also had positive influences on the pectin lyase activity.

The significance of these seconder order equations was analyzed statistically using variance analysis. The ANOVA results of models showed that they could be used to navigate the design field (Tables 3-6). The very low P-value (<0.0001) and the F-value (30.01) of model for the endo pectinase activity demonstrated that it was highly significant within a 95% confidence interval. For the pectin lyase activity, the F-value of model (4.85) indicated that the model was important. The probability of a large Model F-Value due to noise was only 1.07%. A high correlation between the experimental and predicted activity values was indicated by the value of the determination coefficient R^2 for the endo-pectinase activity (0.9643). This showed that the regression model explained the relationship between the endo pectinase activity and the wheat bran amount, $(NH_4)_2SO4$ and yeast extract concentrations well. In addition, this indicated that 96.43% of the variation for the endo-pectinase activity was explained by the wheat bran, $(NH_4)_2SO_4$ and yeast extract concentrations and it could not describe only 3.57% of the variation. The adjusted R^2 of 0.9322 was too high to indicate that the it had a high significance [24]. The adjusted R^2 arranges the R^2 values. If there are a few variables in a model and the sample size is not very large, the R^2 may be clearly higher than the adjusted R^2. The predicted R^2 of 0.7382 and the adjusted R^2 of 0.9322 were in reasonable agreement. It had a partially high determination coefficient (R^2 =0.8136) for the pectin lyase activity. When the R^2 values are less than 0.75, models generally show an inadequate definition of experimental data. The determination coefficient for a good model fit should be at least 0.80 [25]. The coefficient of variation (CV=8.81%) for the endo pectinase activity was relatively low. This revealed that the experiments were more accurate and

reliable. Adequate precision specifies the ratio of signal to noise. It is desired to be greater than 4. In this study, the model ratios for pectin lyase and endo-pectinase were determined as 6.955 and 13.149 respectively. This showed an adequate signal and proved that they can be used to navigate the design space.

Table 3. *ANOVA results for endo- pectinase activity*

Source	Sum of squares	DF	Mean square	F- value	Probability (P) > F
Model	35944.59	9	3993.84	30.01	<0.0001
Residual	1330.98	10	133.10		
Lack of fit	1231.91	5	246.38	12.43	0.0076
Pure error	99.07	5	19.81		
Corrected total	37275.57	19			

R^2: 0.9643, Adj. R^2: 0.9322, Pred. R^2: 0.7382, Adeq. Precision: 13.149

Table 4. *Estimates of the model regression for endo- pectinase activity*

Source	Parameter estimate	F- value	Probability (P) > F
Intercept	174.50	30.01	<0.0001
A	0.29	8.606E-0.003	0.9279
B	-9.20	8.69	0.0146
C	-22.25	50.79	< 0.0001
AB	0.32	6.300E-0.003	0.9383
AC	-3.56	0.76	0.4034
BC	24.27	35.42	0.0001
A^2	-34.07	125.67	< 0.0001
B^2	-5.27	3.00	0.1138
C^2	-24.49	64.94	< 0.0001

Table 5. *ANOVA results for pectin lyase activity*

Source	Sum of squares	DF	Mean square	F- value	Probability (P) > F
Model	3103.18	9	344.80	4.85	0.0107 significant
Residual	710.97	10	71.10		
Lack of fit	680.35	5	136.07	22.22	0.0020 significant
Pure error	30.61	5	6.12		
Corrected total	3814.15	19			

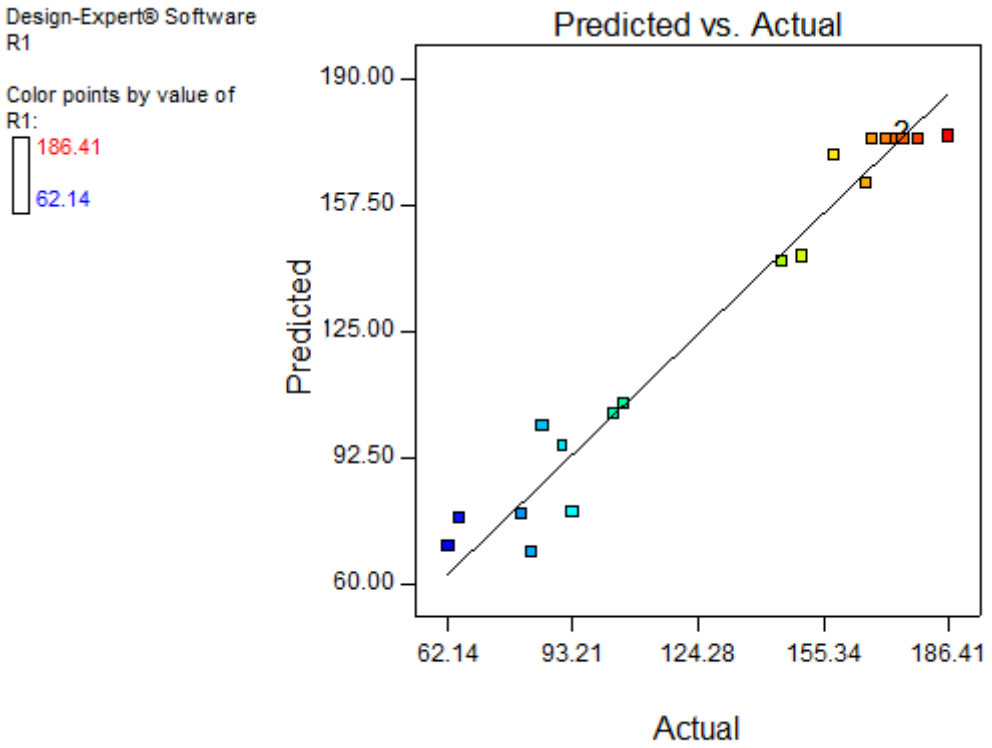
R^2: 0.8136, Adj. R^2: 0.6458, Adeq. Precision; 6.955

Table 6. *Estimates of the model regression for pectin lyase activity*

Source	Parameter estimate	F- value	Probability (P) > F
Intercept	26.26	4.85	0.0107
A	-2.15	0.89	0.3678
B	-4.74	4.32	0.0643
C	-10.72	22.08	0.0008
AB	-2.16	0.53	0.4848
AC	2.30	0.59	0.4587
BC	4.21	2.00	0.1880
A^2	-8.01	12.99	0.0048
B^2	0.26	0.014	0.9082
C^2	-1.00	0.20	0.6623

The significance of the model terms can be revealed by *p*-values [26]. Model terms with p-values less than 0.05 are important. In this situation, *B, C, BC,* A^2 and C^2 are important model terms for endo pectinase activity. The linear and quadratic effects of the yeast extract concentration (*C* and C^2) and the quadratic effect of the wheat bran concentration (A^2) (P<0.0001) were more significant than the other factors. The effect of interaction between $(NH_4)_2SO_4$ and the yeast extract concentration (*BC*) (probability coefficient P= 0.0001) was highly important. The linear effect of $(NH_4)_2SO_4$ concentration (*B*) (probability coefficient P= 0.0146) was significant. The other terms had no significance. *C* and A^2 were significant model terms in the pectin lyase activity. The linear effect of yeast extract concentration (*C*) (P=0.0008) was an important factor. In addition, the quadratic effect of wheat bran concentration (A^2) (P=0.0048) was significant.

Generally, it is important to make sure that the chosen model provides an appropriate approach to the actual system. The adequacy of a model can be judged using the diagnostic plots such as the predicted versus actual value plot (Figures 1a and 2a) [27]. When Figures 1a and 2a are examined, it was understood that there was a linear relationship between the predicted and actual values and models were adequate [25]. The correlation coefficients between the actual and predicted values were 0.9820 and 0.9020 for the endo-pectinase activity and pectin lyase activity, respectively. Higher values of the correlation coefficient justify a perfect relation between the independent variables [24]. The R-value indicates good agreement between the calculated and observed activities in the experimental range. The experimental activity values were in close agreement with the activity values obtained using RSM. The residual plots were investigated using an approximate model. The normal probability and studentized residuals graphs are illustrated in Figures 1b and 2b. The normal probability graph shows whether the residuals follow a normal distribution and in this case the points lie close to a straight line [28] describing the effectiveness of model [29]. As can be seen from Figures 1b and 2b, s-shaped curve did not form. This shows that no response transformation was required, and that there was no obvious issue with normality [28]. Figures 1c and 2c present the perturbation plots that show the effect of each of the independent variables on the endo-pectinase activity (R1) and pectin lyase activity (R2).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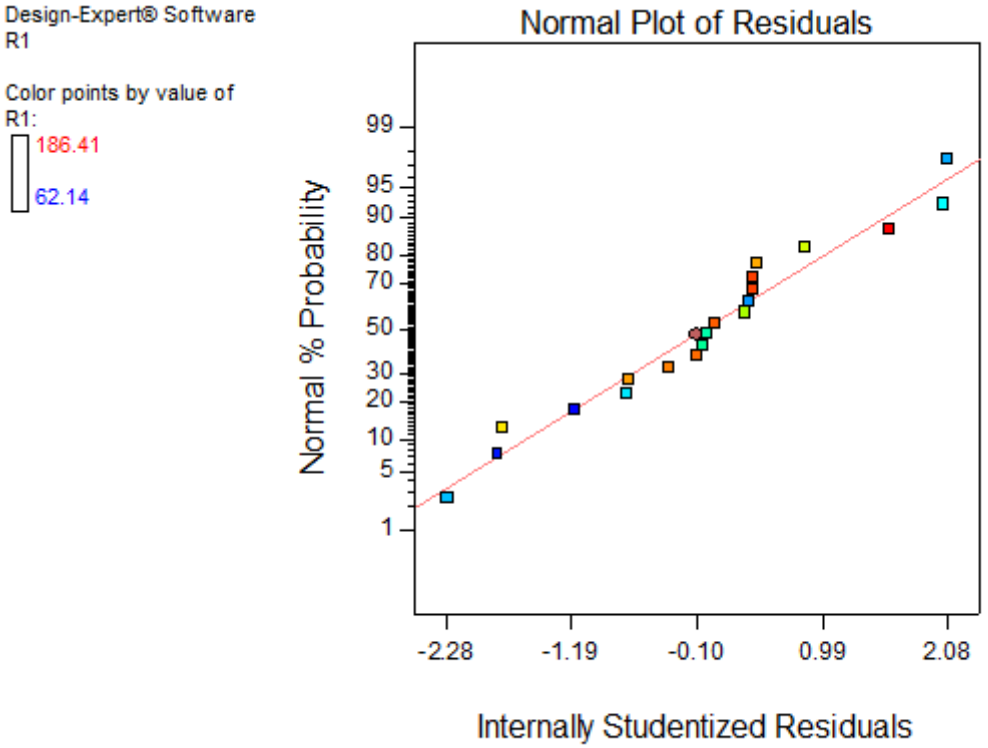
(a)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(b)

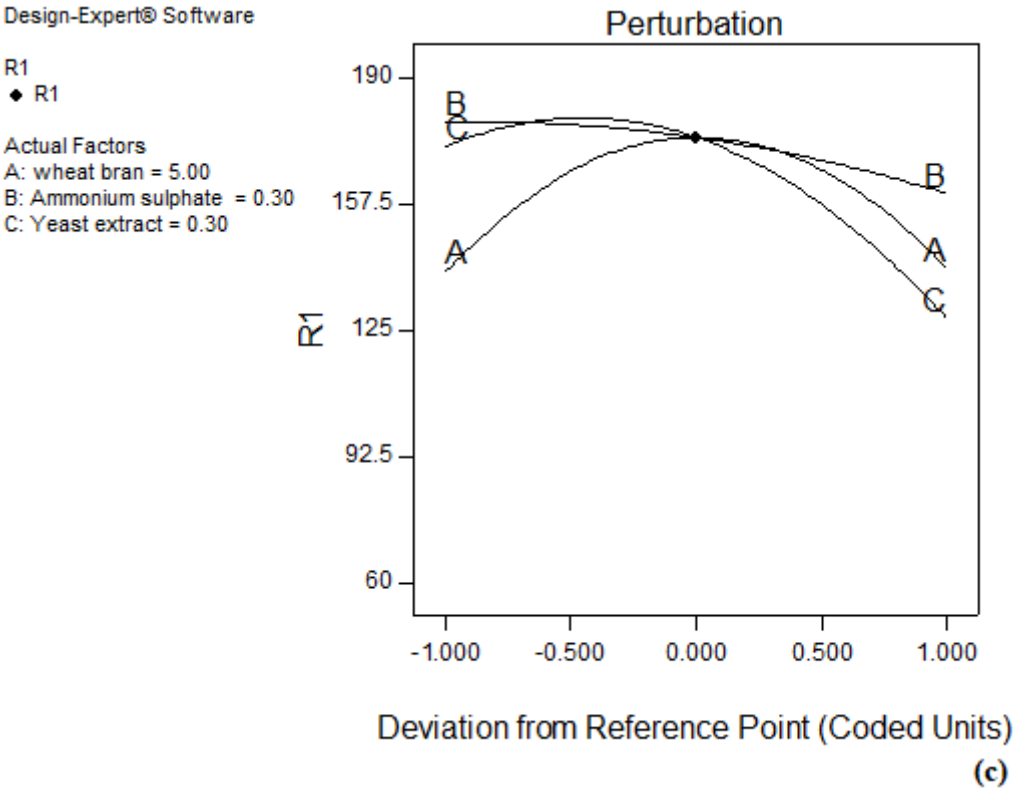
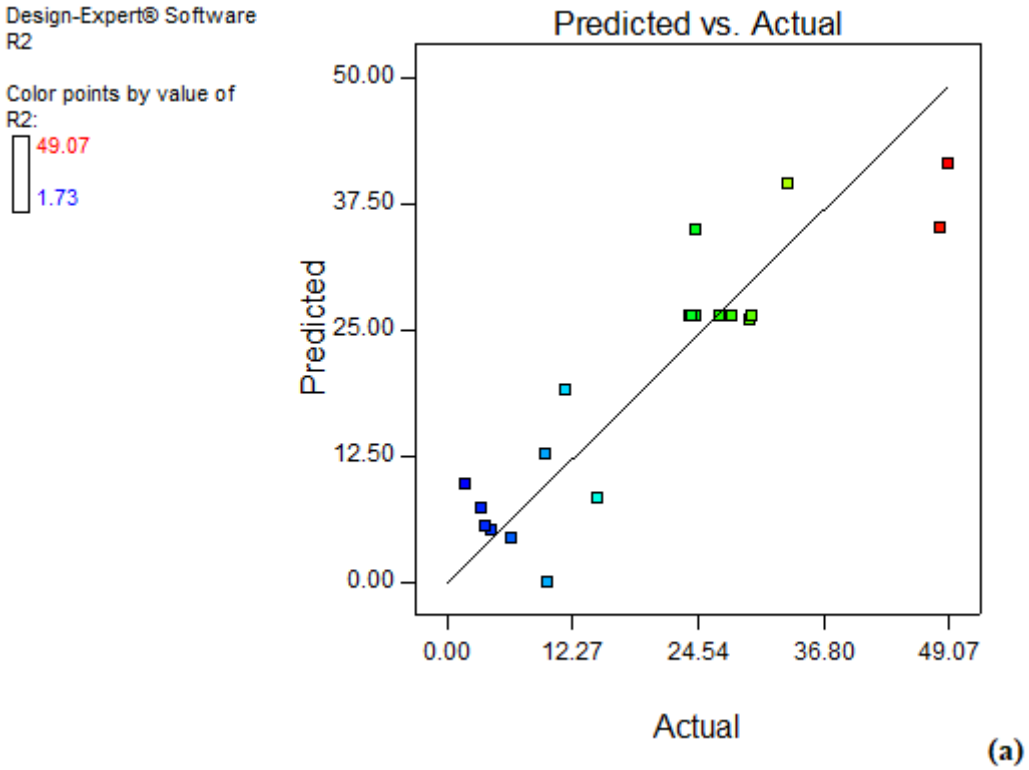
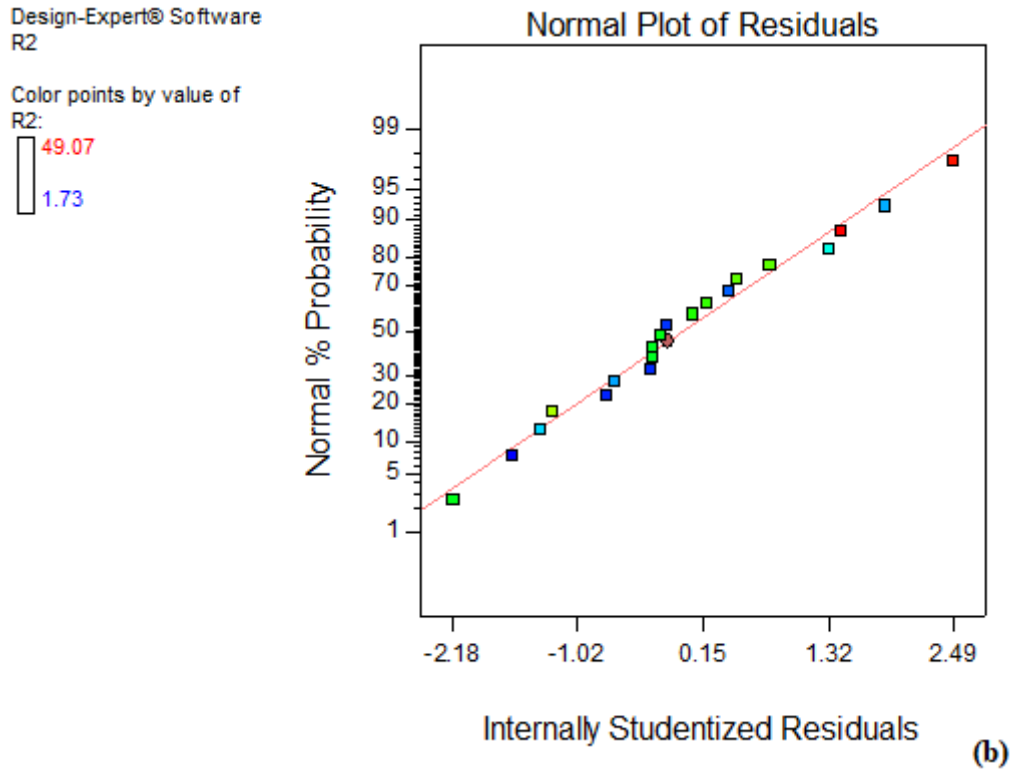


Figure 1. *The actual data versus predicted data (a), the normal probability and studentized residuals graph (b) for endo-pectinase activity (R1) and perturbation plot showing the effect of each of the independent variables on endo-pectinase activity (R1) (c)*





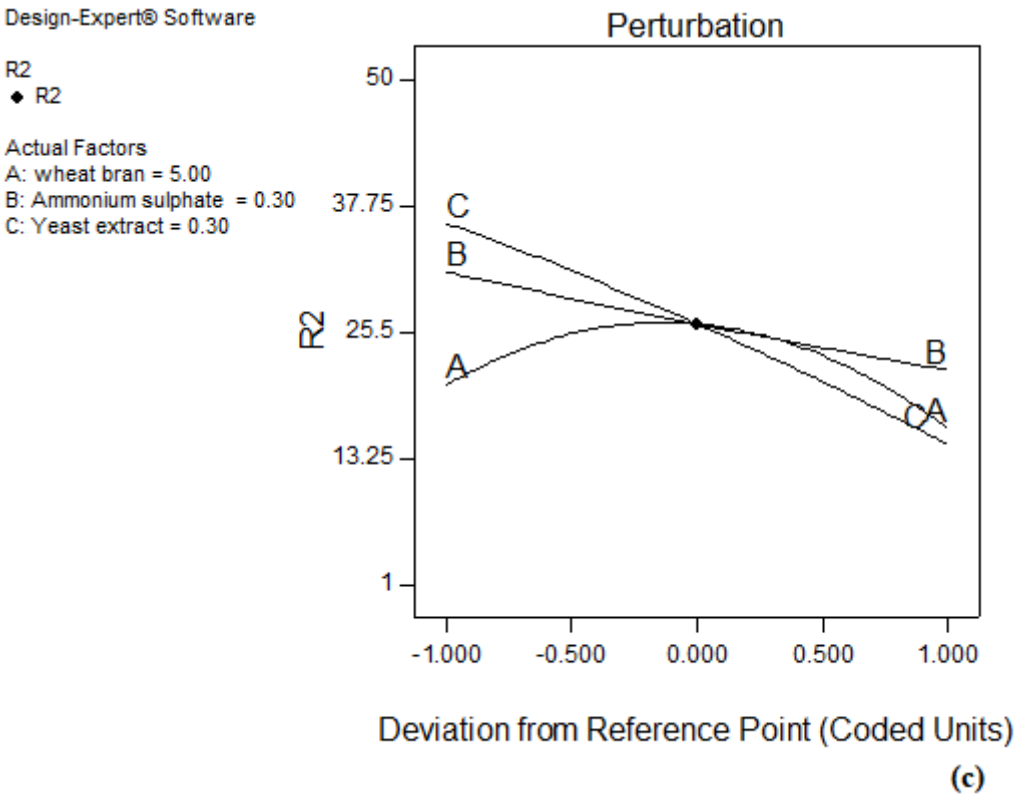


Figure 2. *The actual data versus predicted data (a), the normal probability and studentized residuals graph (b) for pectin lyase activity (R2) and perturbation plot showing the effect of each of the independent variables on pectin lyase activity (R2) (c)*

The regression formula obtained from the RSM analysis can be illustrated with 3-D and 2-D curves. These curves are plotted using a statistically suitable model to reveal the optimum levels of each element required for maximum enzyme synthesis and to comprehend the interaction of media elements [20]. Thus, with these 3-D and 2-D curves, finding the optimal variable concentrations is also very simple and convenient [30]. All plots are presented in Figures 3-8. In all plots, while a variable was maintained to zero (0) level, the effect of the two factors was presented. The pectin lyase and endo-pectinase activities were found to increase when low levels of *B* and *C* and a moderate level of *A* were used. The relatively circular nature of the 2-D plots in Figures 3 and 4 is evidence that there was almost no interaction between *A* and *B* and *A* and *C*. The 2-D curves in Figure 5 showed that there was a mutual interaction between *B* and *C* for the endo-pectinase activity.

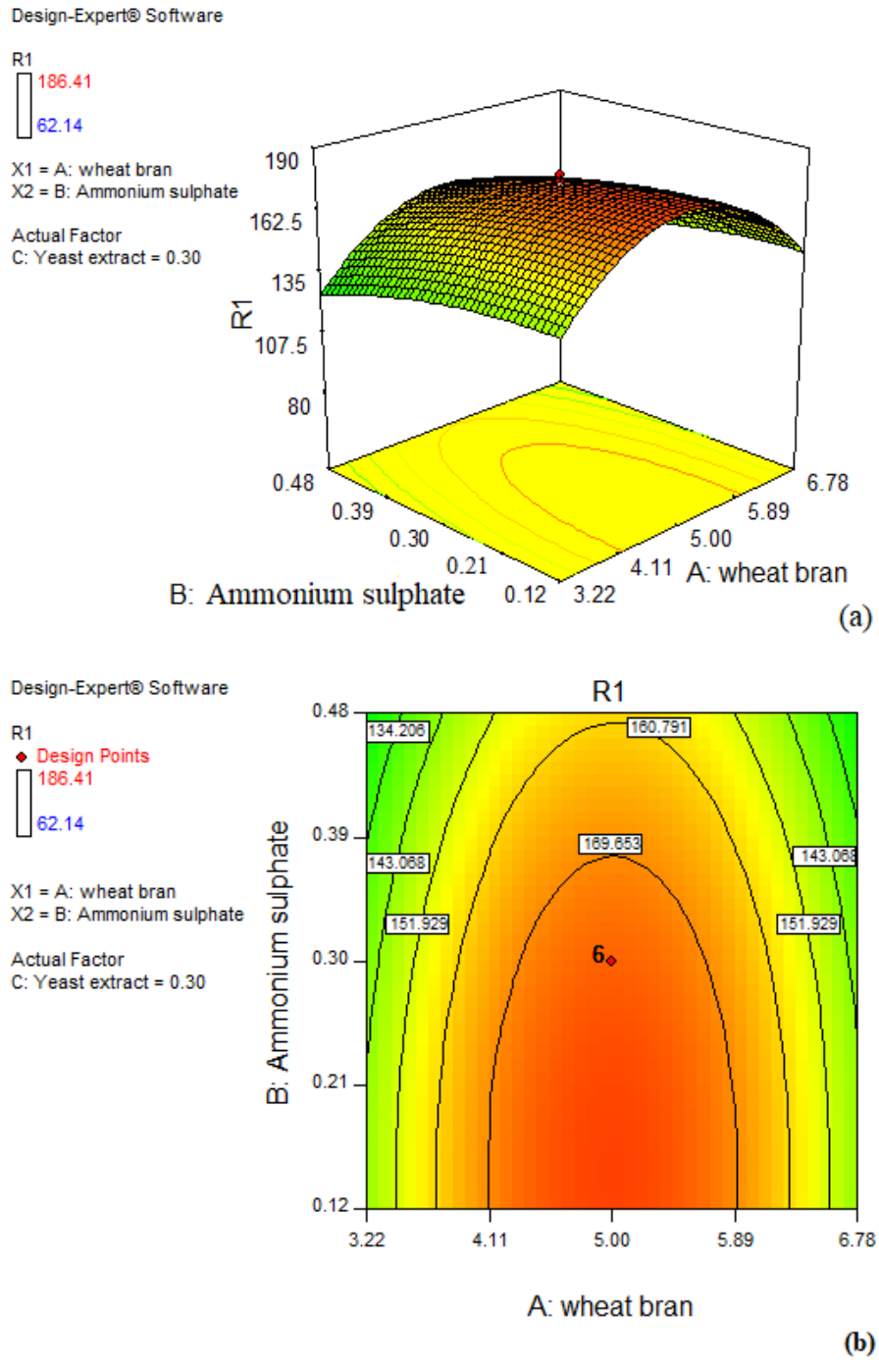


Figure 3. *Response surface plots for endo-pectinase activity (R1)*

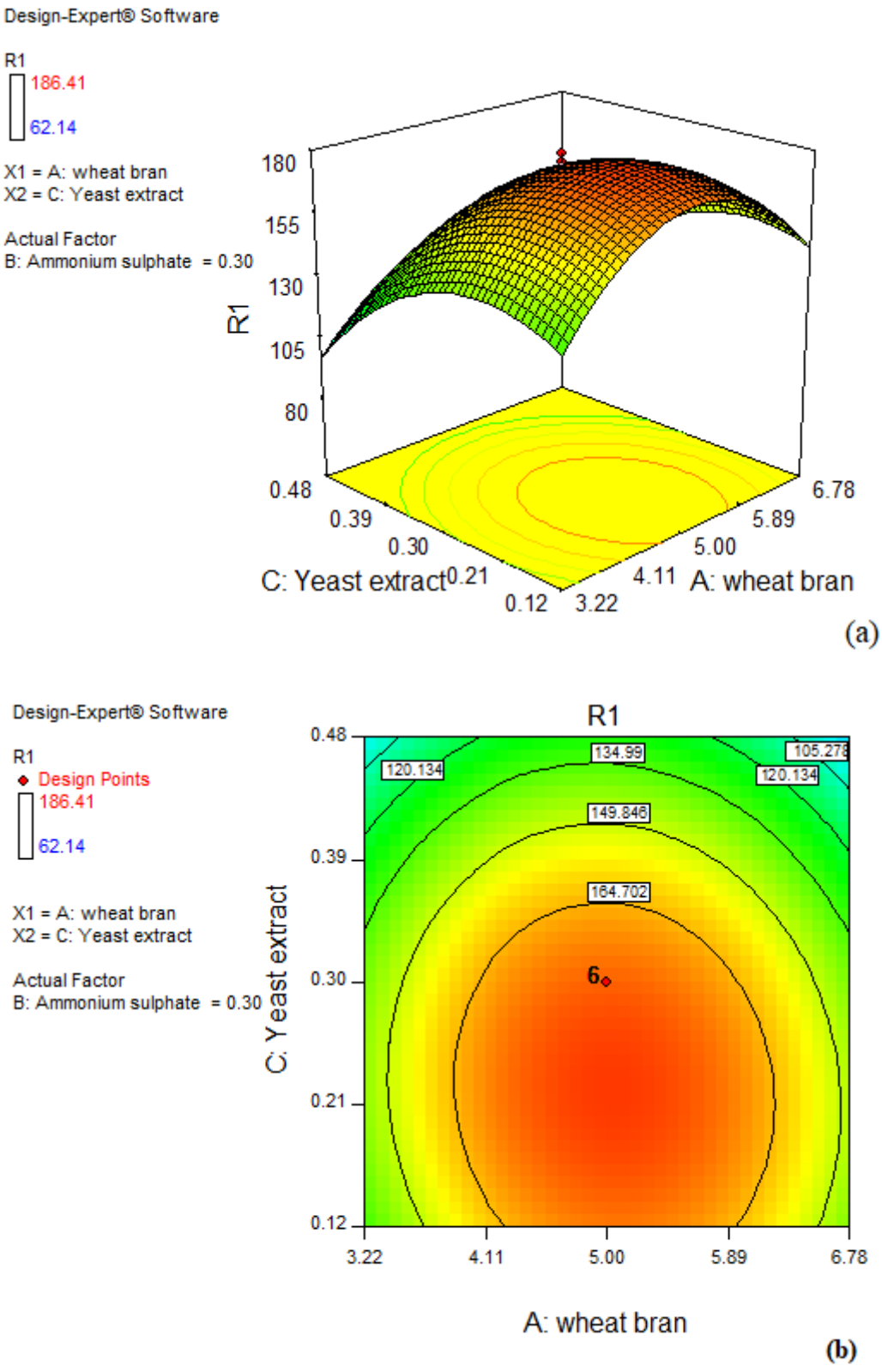


Figure 4. *Response surface plots for endo-pectinase activity (R1)*

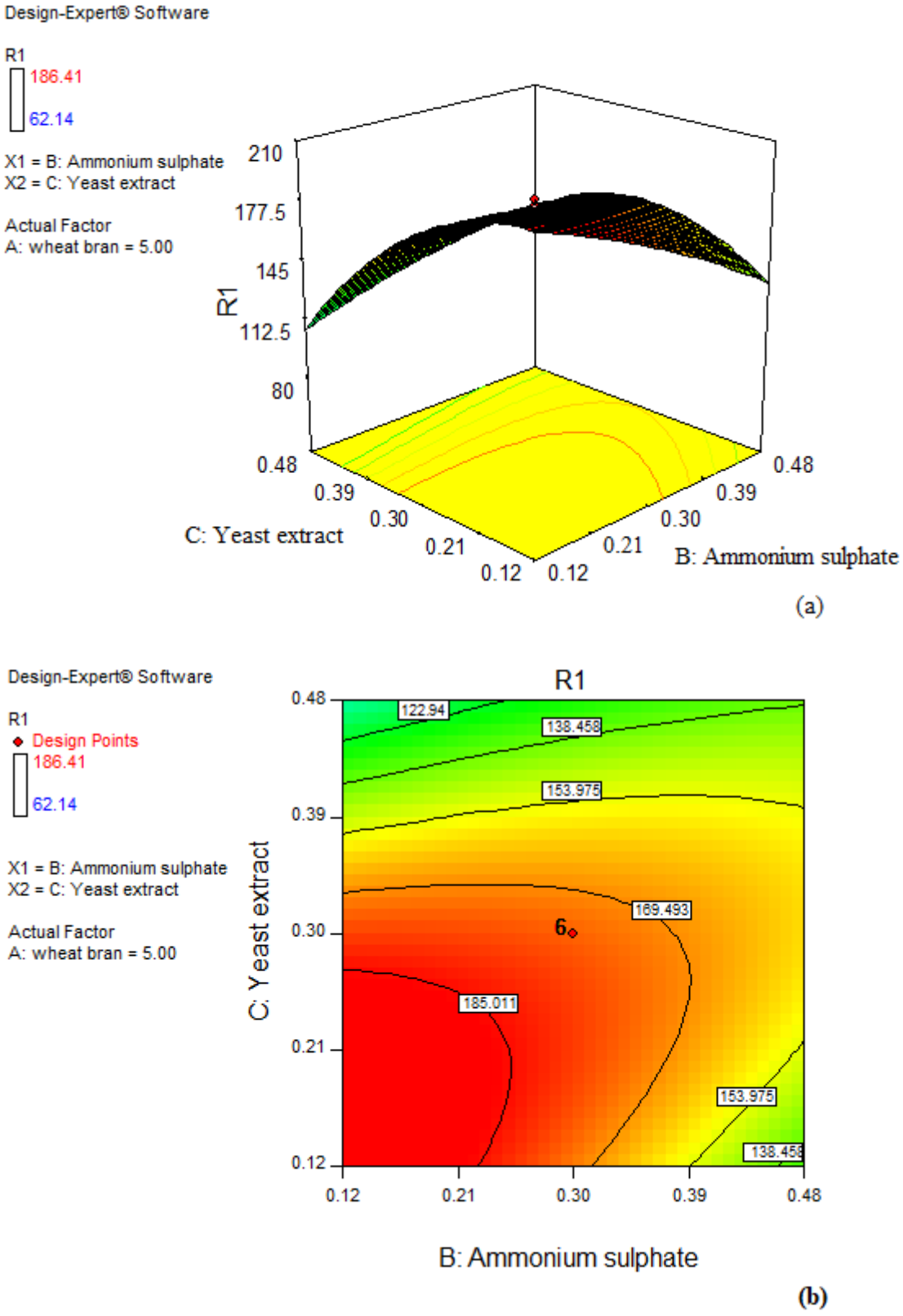


Figure 5. *Response surface plots for endo-pectinase activity (R1)*

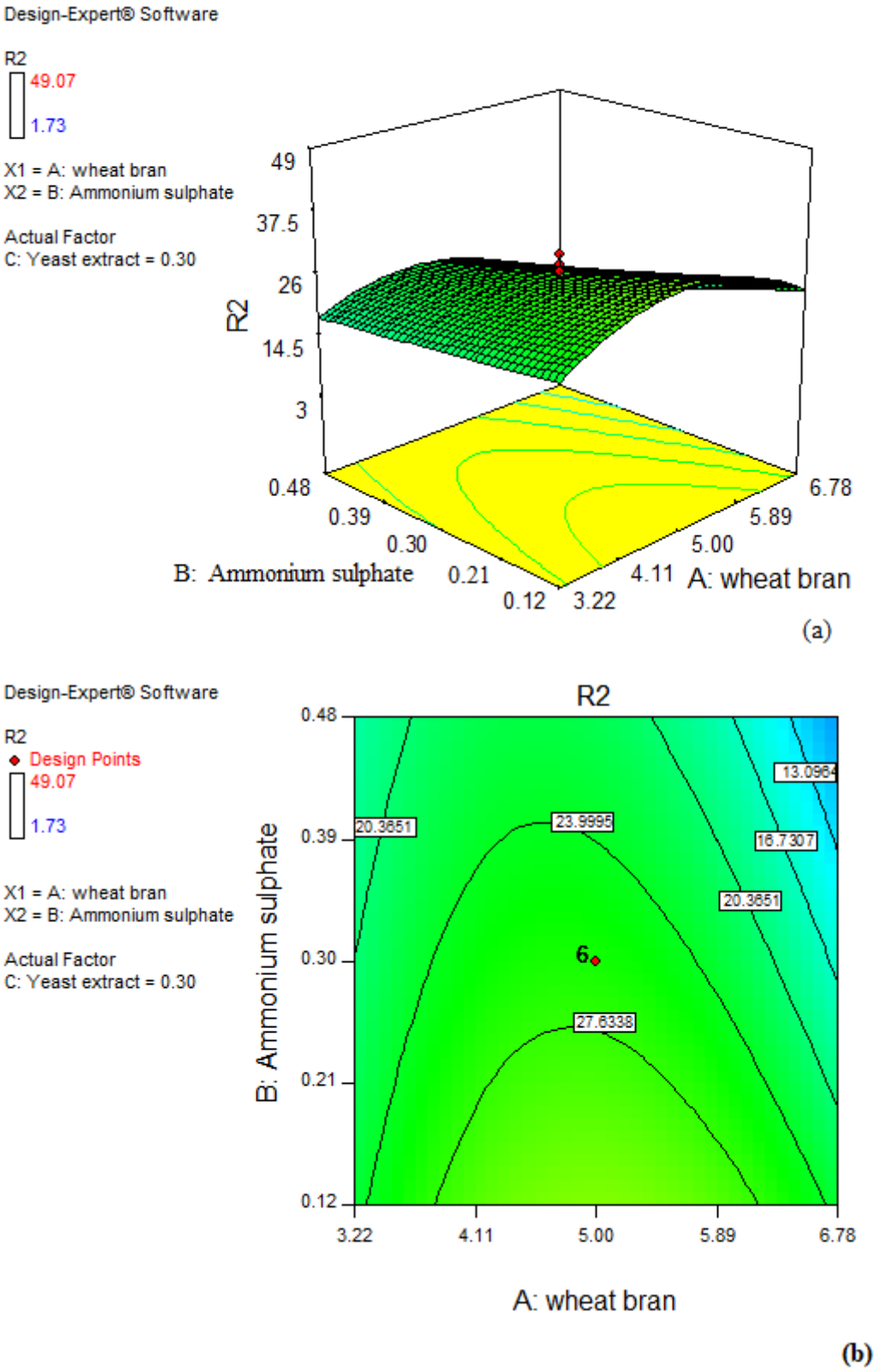


Figure 6. *Response surface plots for pectin lyase activity (R2)*

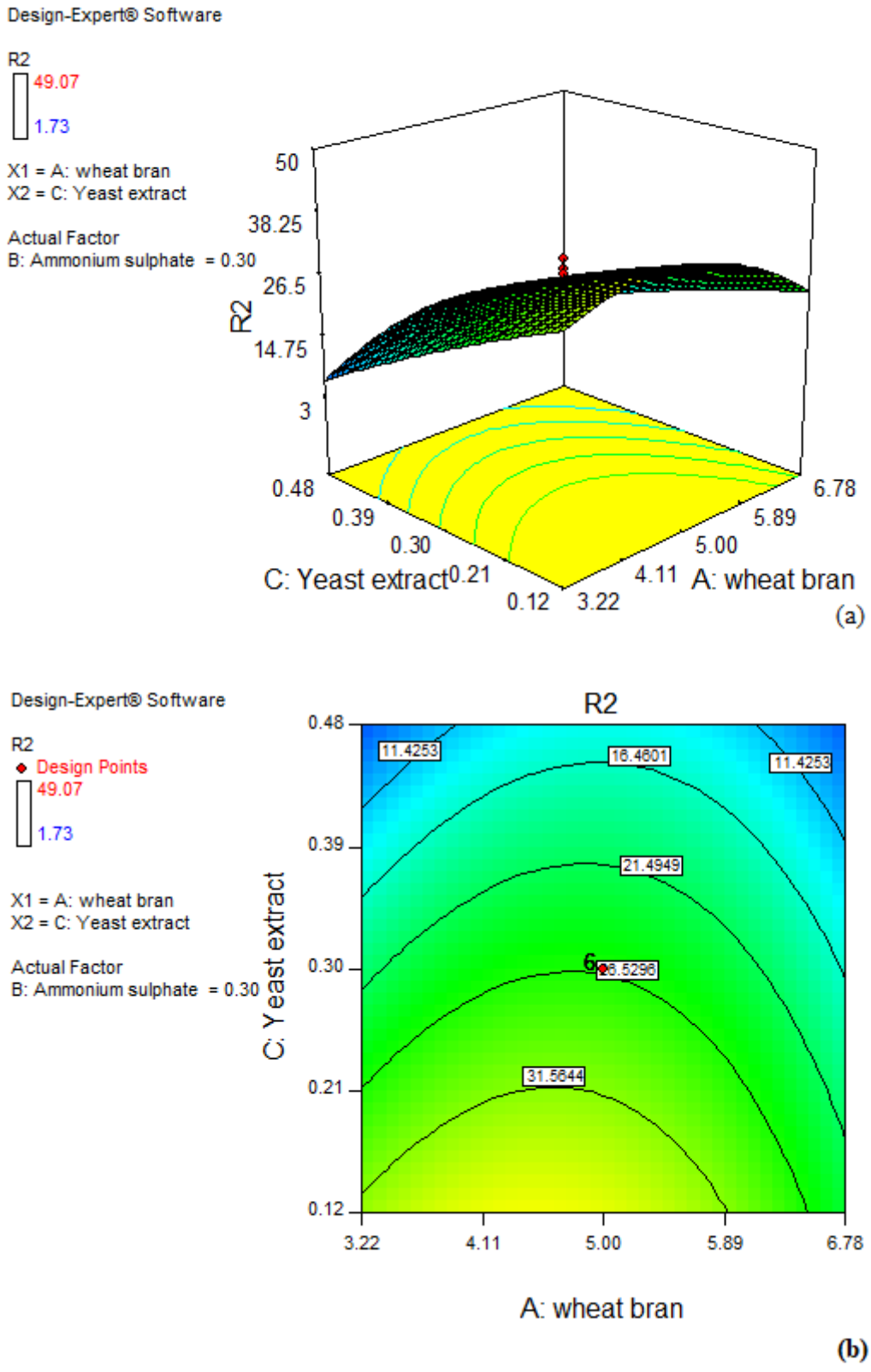


Figure 7. *Response surface plots for pectin lyase activity (R2)*

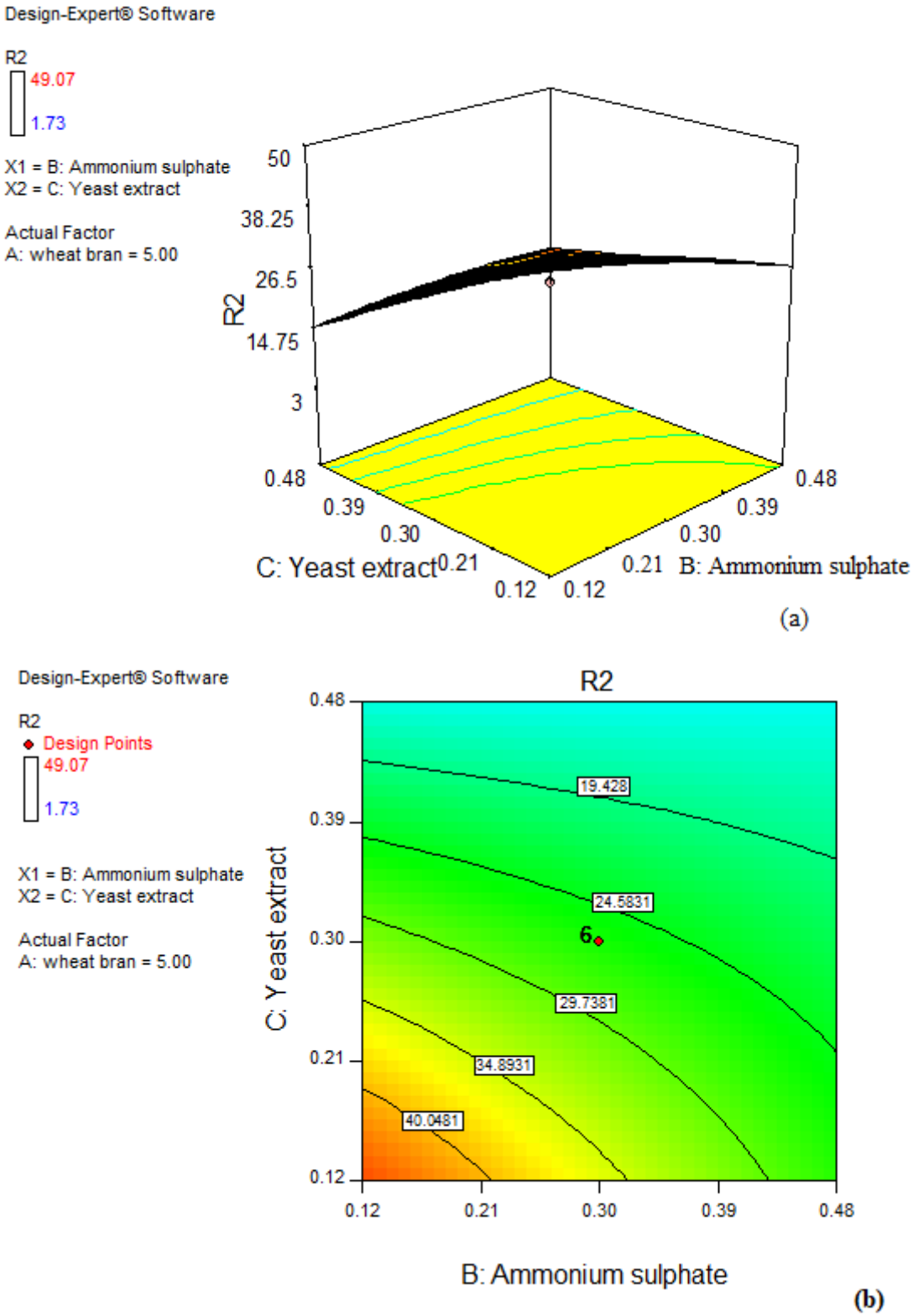


Figure 8. *Response surface plots for pectin lyase activity (R2)*

To obtain the maximum endo-pectinase and pectin lyase activities of 199.21 and 45.37 U/mL, respectively, the optimum conditions were determined as 4.74% w/v wheat bran, 0.12% yeast extract and 0.12% $(NH_4)_2SO_4$ with a desirability function of 0.9798 (Figure 9). The closer the desirability value is to 1 [31], the more suitable the levels of the media components selected to achieve maximum activity.

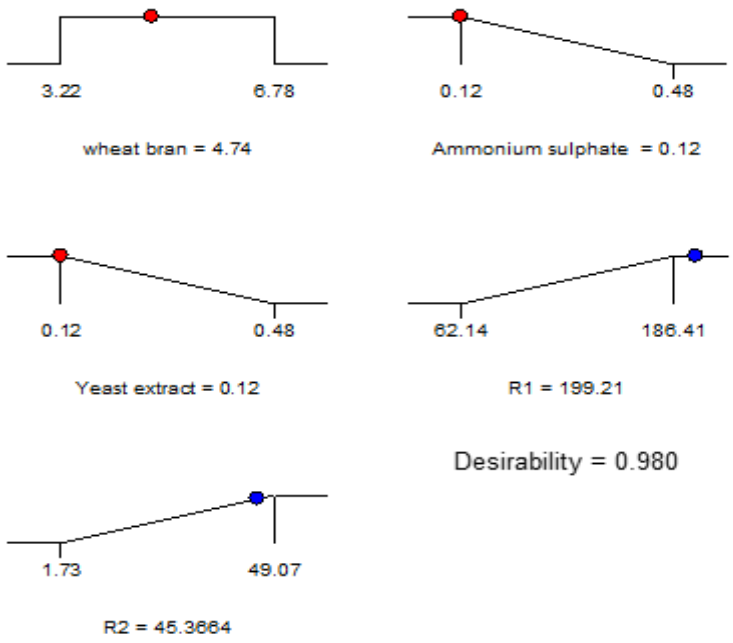


Figure 9. *Desirability ramp of endo-pectinase and pectin lyase production for numerical optimization of three independent variables*

The most important components of bacteria's biological process medium are carbon and nitrogen [32]. The excess wheat bran in the fermentation media reduced the enzyme production. When a low amount of nitrogen was used in the fermentation medium, enzyme production was found to increase. The excessive addition of the nitrogen source can decrease enzyme production. This can be due to the fact that an optimal carbon/nitrogen (C/N) is necessary for maximum enzyme production [33]. The C/N ratio (C/N) is a critical factor that influence cell growth and the yield of the product during fermentation [34]. The C/N ratio used (the highest activities were obtained) in this study was approximately 20 with 4.74% wheat bran and 0.24% nitrogen (0.12% for ammonium sulphate+0.12% for yeast extract=0.24%). In a previous study conducted by Anvari and Khayati [35] it was stated that the highest pectinase production was obtained at a C/N ratio of 10:1 and that a very high or very low C/N ratio provided relatively less enzyme activity. However, enzyme production at a C/N ratio of 10:1 was found to be no different from that of the C/N ratio of 20:1. Thus, it can be said that the tolerable limit of C/N for pectinase enzyme production is not too narrow. An excessive increase in the C/N ratio decreased enzyme production. Nair and Panda [36] determined that the highest pectinase activity was achieved with C/N ratios between 10 and 15.

4. CONCLUSIONS

In this study, wheat bran, which is an agricultural waste, was transformed into value-added products, namely pectin lyase and endo- pectinase by fermentation. The production of pectin lyase and endo-pectinase from wheat bran by *Bacillus pumilus* was optimized by RSM. This methodology has been successfully applied to clutch effects and the interaction between components and. While *B, C, BC,* A^2 and C^2 are important model terms for endo-pectinase activity, *C* and A^2 are significant model terms in pectin lyase activity. The C/N ratio used in this study was approximately 20 with 4.74% wheat bran and 0.24% nitrogen (0.12% for ammonium sulphate+0.12% for yeast extract=0.24%). With the high activity values obtained, it was determined that these enzymes have a great potential especially in industrial applications such as the fruit juice and textile industries.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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