The application of statistical methods to produce pectinesterase , Endo-Pectinase and Pectinlyase through submerged fermentation Using *Aspergillus niger* and optimization of medium

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Abstract : Pectinase enzymes play important role in industrial food applications and also they are an important commercial productions, these enzymes could be used in clarifying juices and wines, fruit oil extraction ,tea and coffee fermentation . Pectinase could hydrolyze the pectin which exists in fruit cell walls; as a matter of fact the hydrolysis could help the yielding of fruit juices. Yeast, bacteria and a great deal of filamentous fungi are the main sources of Pectinase enzymes, and Aspergillus is the most adaptable fungi. Fractional factorial experimental design and central composite design (CCD) have been applied to find seven factors of medium culture on enzymes activity; these factors are as concentration of ammonium sulphate ,potassium dihydrogen phosphate and date pomace ,PH ,total spores ,agitation speed and fermentation time. the results of factorial experimental design indicate that concentration of ammonium sulphate, PH and fermentation time were determined as the most effective factors for pectin esterase and pectinlyase activity ; and the fermentation time was the most effective factor for Endo- Pectinase .it has to be mentioned that all the effects of these factors were analyzed with CCD method .the submerged fermentation of Aspergillus niger were studied to produce Pectinase enzymes ,and also the culture medium were optimized to reach the maximum activity for pectinlyase , Endo- Pectinase and pectin esterase enzymes .desirability function and graphical optimization methods used to find combinations of optimum condition for culture medium for each enzymes and also for three enzymes in the same time .

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Keywords: Pectinesterase, Endo-pectinase, Pectinlyase, Aspergillus niger, Date pomace optimization, enzyme activity

1.Introduction

Pectinase enzymes derived from Aspergillus niger are classified as GRAS and they are mentioned acceptable to be used in food industries (Malvessi et al, 2004). As BE Lafi-Bako et al announced, Pectinase could be separated in three major classes including pectin esterase, depolymeising hydrolytic enzymes and lyases. Several funguses could produce Pectinase enzymes like Fusarium moniliforme ,Botrytis cinerea ,Rhizoctonia solani ,Aspergillus sp and etc ,can produce Pectinases enzymes in which Aspergillus niger is more useful in comparison with others (Kuamar et al, 2006). a great deal of agriculture wastes used as substrate sources for the Pectinases production which these wastes are as wheat bran ,sugar beet pulp ,citrus waste ,pumpkin oil cake ,grape pomace and orange peels ,etc .date pomace could be used in syrup industry ,so it could be used as a cheap substrate source for Pectinase production. According to the results of numerical and graphical methods , the maximum activity for Endo-Pectinase (10.87 u/ml), pectin esterase (3.67 u/ml) and pectinlayse (1.38 u/ml) would be occurred in the same time in the optimized condition including fermentation time (75.27 hour), Ammonium sulphate concentration (%0.35 gr /ml), PH(4.82), total spore (10^5 gr/ml), date pomace (%6), potassium dihydrogen phosphate concentration (%0.8) and agitation speed (250rpm); it has to be mentioned that reduction of fermentation time to 68.27 ,did not make any difference for enzyme activity ,and this time considered appropriate for enzymes production with high enzyme activity and minimum costs .

The aim of present research is to observe the influence of several factors in activity of pectin esterase enzyme in a date pomace substrate, and also optimizing pectin esterase activity in the submerged fermentation production of this enzyme through Aspergillus niger. observed factors are included of fermentation time ,agitation speed , PH, Ammonium sulphate concentration, potassium dihydrogen phosphate, date pomace and total spore ; these factors are studied to find each factors 's effect on Endo-Pectinase, pectinlyase and the activity of pectin esterase enzymes . The desirability function and graphical optimization methods have been also used to find the optimum conditions.

2. Methodology

Several spoiled fruits like apple, orange ,cucumber ,apricot ,strawberry ,tomato ,lemon and grapes were used to prepare microorganisms .growth of fungi in some fruits were so quick, like orange and

lemon whereas the growth was not observed in apple ,and the fungi was observed in orange and lemon in 48 hours .the mentioned funguses were cultured in PDA medium (PH=4.8-5.6) and incubated for five days at 25°C ;the separated funguses were isolated in this medium .enzyme activity could be determined through adding %1 solution hexadecyl trimethyl bromide for 30 minute ,in this case the fungi would grow (Moyo et al, 2003). the largest corona diameter was found for grapes (34 mm), and Aspergillus niger PG5 was selected among several microorganisms that were extracted from spoiled fruits. Morphological trait was used for determining Aspergillus niger spores under microscope in x40view. spectophotometer with 600nm wavelength was used to count the amount of Aspergillus niger spores ,and to regulate the amount of spores , in this case the suspension was diluted with physiological serum solution. date pomace could be a useful substrate, thus it 's dried powder in 70°C could be used in culture medium .the chemical composition of date pomace were analyzed and determined per Iranian national standard including Ash(%3.6),lipid (%1.5), fiber (%24.87), protein (%7.9) and dry material (%96.7). The culture medium composition (date pomace powder, Ammonium sulphate and potassium dihydrogen phosphate) scaled in a ml within fixed PH and autoclaved for 15 minute in 121°C and 15 psi pressure .in order to determine the effective factors on enzyme activity in the first step , several compositions of these factors as an experimental design were prepared for culture medium .according to table 2 , the factors including PH(3.5-5), total spore amount ($10^5 - 10^7$ $spores/ml$), KH_2PO_4(0.4-0.8 g/100ml), $(NH_4)_2SO_4$ (0.1-0.3 g/100ml),agitation speed (150-250rpm), date pomace (4-8 g/100 ml), and fermentation time (24-48).in the second step, significant factors were selected and new compositions of these factors were prepared for culture medium as anew experimental design (table 3).

The viscometry method was used to describe the activity of Endo- Pectinase enzyme, and %2 pectin solution within PH=5.5 used as the substrate . the unit of Endo- Pectinase was determined as the amount of enzyme in which the viscosity of solution could be reduced to %50 in10 minute .also the activity of enzyme is specified as unit in a milliliter culture medium filtrate (U/ml)(Loera et al ,1999).To define the pectin esterase activity , Two milliliters of culture medium filtrate were added to 10 ml of %0.5 pectin solution and NaCl molar .PH was adjusted in 4.5 through NaoH 0.1 molar ,then the mixture incubated in 30 °C for 60 minute .enzyme activity was determined per unbounded Carboxyl 's measurement through NaoH 0.02 normal titration . The pectin esterase activity unit defined as release amount of enzyme through a milliequivalent Carboxyl in a minute .enzyme activity specified as a unit in a milliliter culture medium filtrate (U/ml)(Maldonado et al ,1998).

Main body

in the first step ,effects of seven factors were studied with a two-level fractional factorial selected for experimental design with 32 compositions, all these could be observed in table one .the results of first experimental design were analyzed with half normal plot method ,and the factors out of the curve were selected as significant factors that their variations were no random errors .factors with significant effect on pectin esterase enzyme activity which selected for second step were fermentation time ,PH ,and $(NH_4)_2SO_4$, and the fermentation time just has been used for Endo- Pectinase. to determine the specific effect of these three factors , a central composite design (CCD) with 24 experimental trials containing central points (table 2) .in CCD analysis, the data is fitted to a quadratic equation .regression method and lack of fit test used to valuate this model.

Table 1. Establishing the experimental domain

Symbols	Independent variables	Coded levels	
		-1	+1
X1	Fermentation time (h)	24	48
X2	Agitation speed (rpm)	150	250
X3	pH	3.5	5
X4	$(NH4)_2SO_4$ (g/100ml)	0.1	0.3
X5	KH_2PO_4 (g/100ml)	0.4	0.8
X6	Date pomace (g/100ml)	4	8
X7	Total spore (spores/100ml)	10^5	10^7

Table 2. Five levels Central Composite Design (CCD)

Run	Ammonium Sulfate (mgml−1)	Time (h)	pH
1	0.253	54	4.8
2	0.447	78	4.8
3	0.447	54	6.2
4	0.35	69	5.5
5	0.252	78	6.2
6	0.35	69	5.5
7	0.35	69	5.5
8	0.447	54	4.8
9	0.253	78	4.8
10	0.35	69	5.5
11	0.253	54	6.2
12	0.447	78	6.2
13	0.518	69	5.5
14	0.182	69	5.5
15	0.35	69	4.3
16	0.35	69	6.7

17	0.35	69	5.5
18	0.35	48	5.5
19	0.35	69	5.5
20	0.35	90	5.5
21	0.35	90	5.5
22	0.35	90	5.5
23	0.35	90	5.5
24	0.35	90	5.5

Micro-organisms (or microbes) are literally microscopic organisms, which can only be seen properly with the aid of a microscope. These include bacteria, microscopic fungi (moulds) and protoctists . Although viruses, which are even smaller than bacteria, are generally considered to be non- living entities, they might also be included here as they are important disease-causing agents. Micro-organisms are the most numerous organisms in any ecosystem. Ecological role of these enzymes could be in plants and vegetable's colonization and stability. As a matter of fact, the enzyme within hydrolysis of plant fibers could produce different types of sugar from plan cells, and this could be used for the funguses to be hydrolyzed more .meanwhile , funguses could use the pectin as the only source of carbon which this shows the most complex system of enzyme . Pectinase enzyme moreover the decay and softening, plays important role for virolans , and funguses could be found in the fruit decay .according to mentioned factors, fruits within pectin are a good source for the funguses separation generating from pectinase .the Ruthenium red method has been used for the observation of pectinase enzyme. The separation from spoiled fruits and studying the pectinase production of molds in a particular environment is the issue of the research.

Figure 1 (A) creation of the light corona through produced pectinase in 13 funguses sampling in two repetitions

RR is a stereoselective stain for pectic acid, insofar as the staining site occurs between each monomer unit and the next adjacent neighbor. The most common methods to separate the microorganisms generating pectinase are as follows: PDA measurement, viscosity measurement. In this study, 13 fungi samples were incubated in cup-plate environment, as a result the enzyme activity and method was utilized as following. To separate the funguses, they were cultured in medium for 6 days in 30**C°,** and they were incubated in slant medium, and the SDA incubation was accomplished on each fungi sample.post 6 days, 5 ml water was added to each slant and vertex was prepared. from the obtained suspension, one ml including $10^3 - 10^4$ *spor* was

added to 20 ml of enzyme production environment, and they were incubated for 6 days in 30 **C° .**post the end of incubation period , the cultured funguses was used ,and cup plate was filtrated and the filtrated medium was provided for doing test including %1 pectin ,and 6 ml diameter holes were created in the medium. Each cup-plate was filled with 100ml Aquapura for 24 hours in 32 **C°** of 100 ml enzyme, and each of holes was covered with 5 ml Aquapura ,and the Aquapura was removed after 10 minute .the light corona diameter of HCI was incubated, then the medium surface was measured for each sampling .the result of corona diameter within two repetitions is as following:

Sampling code	The first Corona diameter	The second Corona diameter	Mean	Standard deviation
001		1.2	1.2	
002	1.7	1.5	1.6	0.14
003	2.1	2.1	2.1	0
004	1.6	1.6	1.6	0
005	1.7	1.7	1.7	0
006	2	1.8	1.9	0.14
007	2.5	2.5	2.5	0
008	2	2.2	2.1	0.14
009	0	0	0	0
010	2.3	2.35	2.325	0.03
011		1.2	1.2	
012	2.5	2.2	2.35	0.2
015	1.6	1.75	1.67	0.1

Among the samplings, the seventh sample has shown the most activity of pectinolytic .according to the obtained results, the samples based on the pectinolytic activity , could be divided into three classifications :

Sampling code	Corona diameter	pectinolytic activity
012,010,008,007,003	2	Good
015,006,005,004,002	1.5-2	Ordinary
011,009,001	< 1.5	weak

These three activities were chosen for doing the Ruthenium red test. Aspergillus niger in two superior samples such as 009-7 and 009-8 within a subculture were cultured and were incubated for 5 days in 30 C°, and the incubation of cZapec of each fungi samples were cultured in medium. Then, each sample was added in agar medium including %0.05, and they incubated for 4 days in 30 C°. Post the end of incubation period, %0.05 Ruthenium red solution was added to each 10 ml medium, and it incubated for one hour in room temperature .finally, Aquapura was used for the decolorization .Around the colony, each three samplings of red corona within light corona could be observed in figure 1.

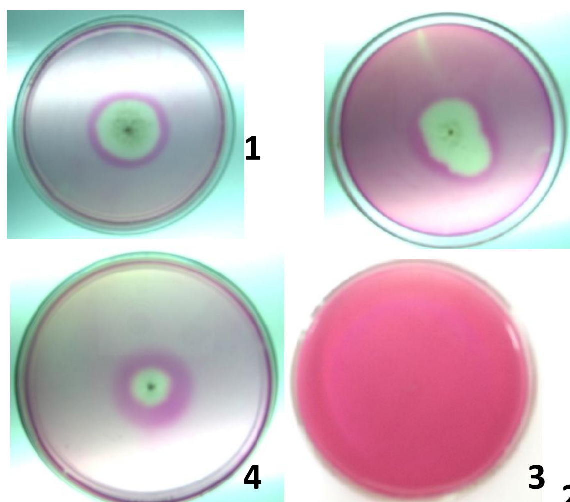


Figure 1 (B) – The creation of light corona in Ruthenium red test for each three sampling such as 009-7 ,009-8 and Aspergillus niger in comparison with the negative sampling

Pectinase enzyme could hydrolyze the pectin in medium in which adding Ruthenium red solution would create a light colony around colonies in which this could lead to positive result .

3. Discussion and results

Fractional factorial experimental design and central composite design results (CCD) Pectin esterase. According to the fractional factorial experimental results, the effect of factos on pectin esterase could be described through equation 3 . the variables used in this equation are coded values of origin variables included fermentation time (X1), agitation speed (X2), PH(X3), $(NH_4)_2SO_4$(X4), KH_2PO_4(X5), date pomace (X6), and total spore (X7). All variables have a linear effect on enzyme activity in their used ranges .for this model,R^2 is almost 0.99 which this amount indicates the adaptability and the model predictions in comparison with real values shows the good correlation (fig 1) .SAS software was used to analyze the results,and the relative effects of all factors were compared with each other (fig 2). According to the Pareto chart ,the fermentation time had have the most effect on enzyme activity ,and fermentation time was followed through concentration of potassium dihydrogen phosphate, Ammonium sulphate ,agitation speed , PH , date pomace and total spore .

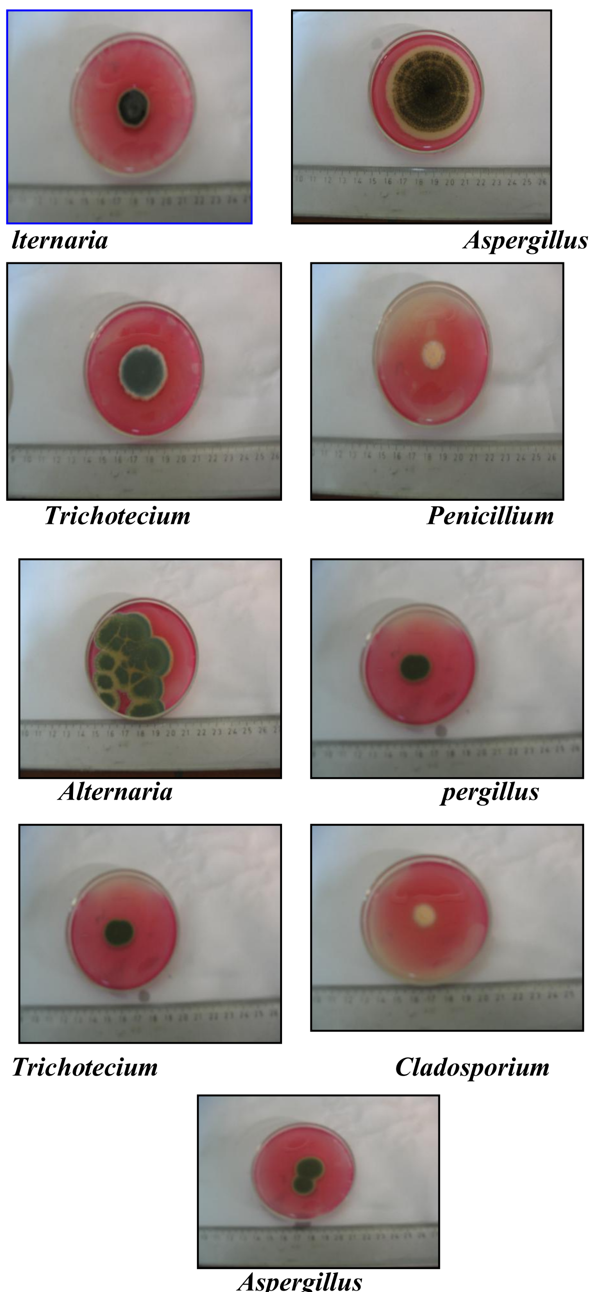


Figure 1 (C) - Model prediction of pectin esterase activity and real values of comparison

Pectin hydrolysis within a light region around colony would get a positive result.

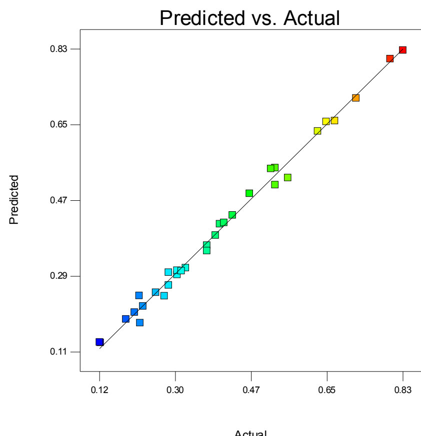
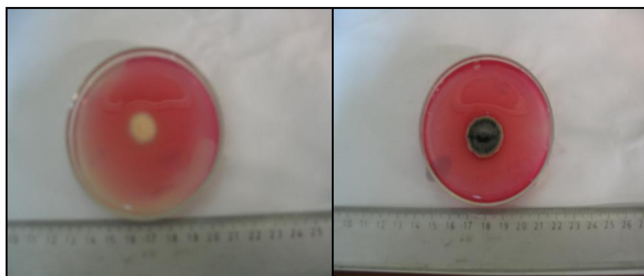


Figure 2 -The Pareto chart shows the comparison between effects of seven factors on pectin esterase activity including A= fermentation time ,B= agitation speed, C= PH, D=$(NH_4)_2SO_4$, E =KH_2PO_4 , F=date pomace , G= total spore



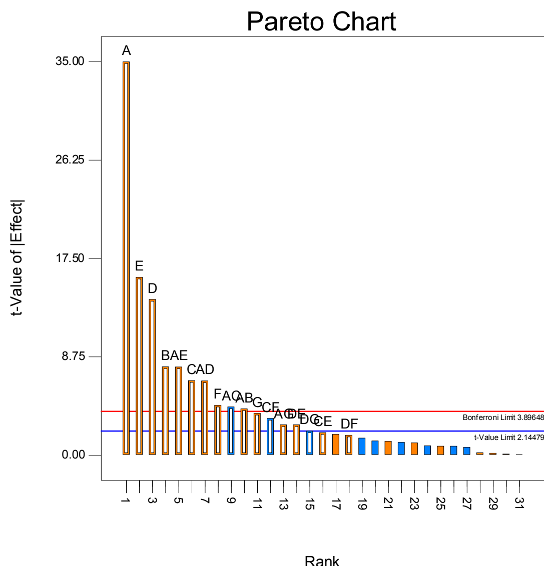


Figure 3. Fermentation time

Fermentation time has the most effect on pectin esterase activity ,and in 24-48 hours of fermentation time , the activity of pectin esterase would increase .Increase of the ammonium sulfate concentration in 0.1 – 0.3 gr / ml region would increase the enzyme activity (figure 3) and the highest pectin esterase activity occurs in 48 hours and %0. 3 concentration of ammonium sulfate. Agitation speed has direct effect on pectin esterase activity, where its highest level occurs in high speed agitation (250rpm) and 48 hours. agitation affects enzyme activity because of its effect on heat and mass transportation and also on fungi morphology , could have positive effect on enzyme activity .the effect of agitation in 48 hours is more than 24 hours ,because as fermentation increases, fungi population grows as well ,and increase of agitation rate would produce more nutrient and oxygen for them. Palil showed positive effect of PH on pectinase activity (Palil et al , 2006) ; and also the increase of PH from 3.5 to 5 would increase the enzyme activity .the increase of date pomace from 4 to 8 gr/ml would have insignificant effect on enzyme activity ,and 10^7 spores per 100 ml in 48 hours fermentation time could be as the highest enzyme activity ,and patil reported the same results as well.In the second step ,three variables such as Ammonium sulphate concentration (0.182-0.45 mg/ml) , PH (4.8-6.12) , fermentation time (48-90 h) were used with a five level experimental design which it 's domain could be seen in table 3.

ANOVA analysis of experimental results shows insignificant effect of PH on enzyme activity in its studied span (4.82 – 6.18). The only effect would be occurred while there is interaction with Ammonium sulphate concentration. the increase of Ammonium sulphate concentration up to %0.35 point in each level of PH would increase the enzyme activity, whereas post this point, the enzyme activity would decrease . although PH has insignificant effect on enzyme activity in 4.82 -6.18 regions ,but it 's interaction with ammonium sulphate concentration would affect pectin esterase activity in %.25 of ammonium sulphate concentration, and the increase of PH from 4.82 to 6.12 would decrease the enzyme activity .while the ammonium sulphate concentration is equal to %0.45 ; in this case the increase of PH would have inverse effect ;thus the increase of ammonium sulphate concentration would not have any effect on enzyme activity and it would just affect the trend of PH effect (figure 3).figure 4 depicts the reponse surface plot for the effect of fermentation time on enzyme activity and it 's interaction with ammonium sulphate concentration . as observed , the increase of fermentation time up to 75 hours would increase the activity of pectin esterase ,and then the activity of pectin esterase would decrease .

Figure 3- response surface plot reveals the effect of PH and ammonium sulphate concentration on pectin

esterase activity (date pomace =g/100ml and other factors are in top levels from first stage design).

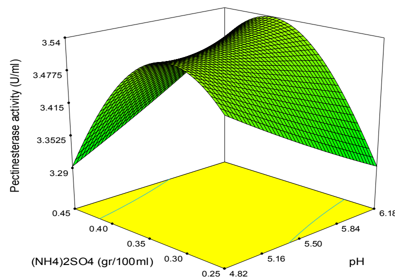


Figure 4- response surface plot reveals the effect of ammonium sulphate concentration and fermentation time on pectin esterase activity (date pomace =g/100ml and other factors are in top levels from first stage design)

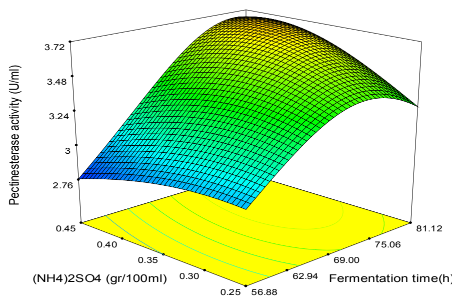


Figure 5-model prediction of Endo-pectinase Activity and real values comparison Endo-pectinase

According to the experimental results, the effect of factors on Endo-pectinase could be expressed through equation 5, in which the comparison of model and real values shows the desirability (figure 5). As shown in pareto chart (figure 6), the fermentation time , concentration of potassium dihydrogen phosphate ,ammonium sulfate ,agitation speed and date pomace have respectively the most effect on the activity of Endo-pectinase enzyme .

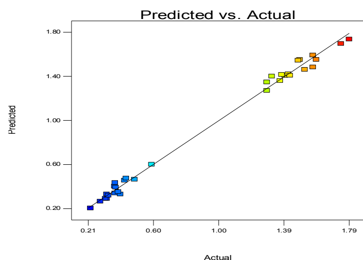


Figure 6-the pareto chart shows the comparison between effects of seven factors on Endo-pectinase including A= fermentation time, B= agitation speed, C= PH, D=$(NH_4)_2SO_4$, E =KH_2PO_4, F=date pomace, G= total spore

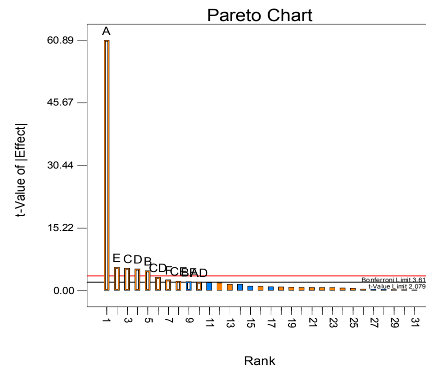


Figure 7- the effect of fermentation time on Endo-pectinase activity

According to the experimental design results, fermentation time has the major effect on Endo-pectinase activity ; increase of ammonium sulphate concentration (%0.1 to %0.3) during 24- 48 hours could increase the activity of enzyme ; the effect of ammonium sulphate in first 24 hours of fermentation time has the least effect on enzyme activity ,and enzyme production depends on the kind of nitrogen source. potassium dihydrogen phosphate also has the positive effect on Endo-pectinase activity in range of %0.4 to %0.8 . many studies reported positive effect of ammonium sulfate concentration on pectinase activity ,and the best concentration of enzyme production is in the range of %0.2 to %0.65.increase of agitation speed (150 to 250 rpm), PH(3.5 to 5)and date pomace concentration (%4 to %8) have low positive effect on Endo-pectinase activity , and fermentation time is the only effective factor on Endo-pectinase activity .

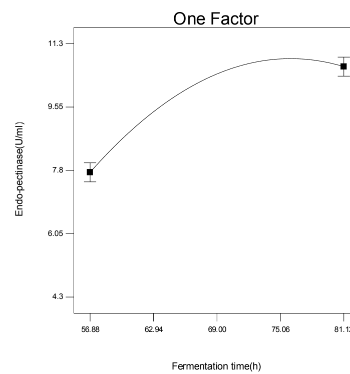


Figure 8-model prediction of Pectinlyase activity and real values of comparison

Pectinlyase

According to the ANOVA analysis ,some factors had no effect on pectinlyase activity ($p > 0.05$) in the observed range such as PH ,agitation speed and total spores .some factors interaction also

were insignificant on this enzyme activity like the interaction between PH with ammonium sulfate ,fermentation time with ammonium sulphate and fermentation time with date pomace concentration .the results of ANOVA analysis showed that the lack of relevancy for this equation was insignificant ,and the desirability has been shown in figure 8. Fermentation time has the major effect on the Pectinlyase

Activity like other two enzymes as shown in pareto plot (figure 9).other factors are sorted in this plot as their important effect on enzyme activity includes the potassium dihydrogen phosphate, ammonium sulphate , date pomace,etc.

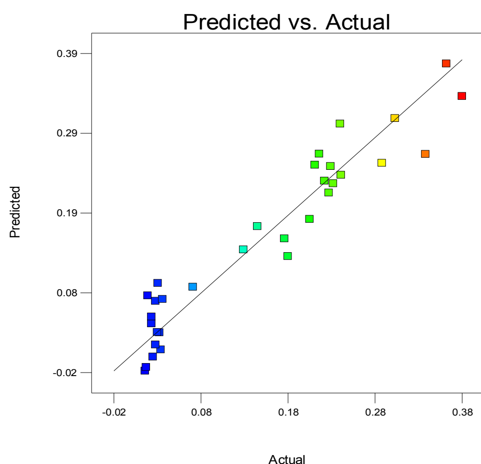


Figure 9-the pareto charte shows the comparison between the effects of seven factors on Pectinlyase activity including A= fermentation time, B= agitation speed, C= PH, D=$(NH_4)_2SO_4$, E = KH_2PO_4, F=date pomace, G= total spore

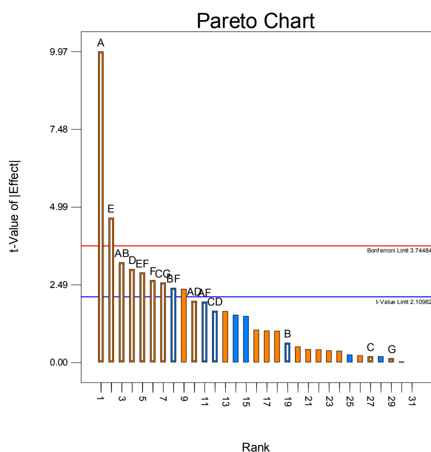


Figure 10-fermentation time effect on Pectinlyase activity

According to the model and experimental design results, potassium dihydrogen phosphate concentration has positive effect on Pectinlyase activity in range of %0.4 to %0.8 and most activity occurs in %0.8 of this factor and 8 gr/100ml of date pomace concentration . ammonium sulphate also has the linear effect on enzyme activity in range of %0.1 to %0.3 . ANOVA analysis shows that fermentation time, PH and ammonium sulphate have positive effect on pectinlyase activity ,and this effect has been shown in equation 8 via CCD method in which R^2 =0.856 , this shows the desirability function for the activity of enzyme prediction .according to the effect of fermentation time in figure 10 , the activity of Pectinlyase

Would increase until 75 hours after fermentation time, and after that it would reduce . ammonium sulphate has a quadractic effect on Pectinlyase activity ,so the increase till %0.35 would increase the enzyme activity , whereas the activity would decrease after this point (figure 11).inversely ,PH has an infinitive effect and it 's interaction with time and ammonium sulphate had no effect on Pectinlyase activity.

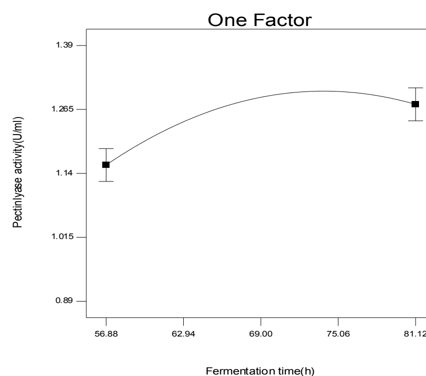


Figure 11- fermentation time effect on Pectinlyase activity

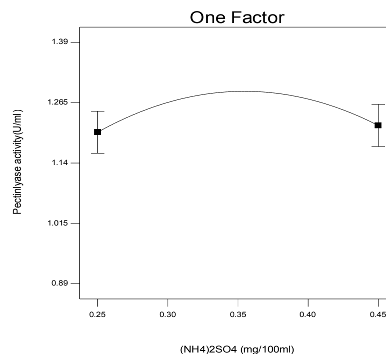


Figure 12-PH effect on Pectinlyase activity

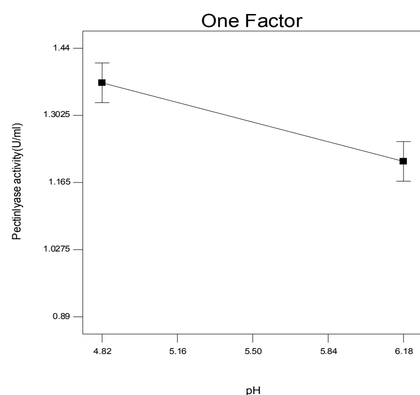


Figure 182-One factor

Optimization

Desirability function method

Desirability functions (numerical method)are such useful method for optimizing production process in the industry .this method was presented by Harrington (Harrington, 1956), and the aim of this method is to find particular conditions of variables which provide high desirability for related function response .desirability value ranges from zero to one, and it 's top value shows the best condition to reach the goal. If the aim of several responses optimization be same, desirability of each response should be determeid and the geometric mean will be used as a index to reach the aim (Alizadeh et al, 2005), so desirability function is used for each enzyme to find the optimum condition sepeareately.

4. Conclusion

While funguses populations grow, they would need more nutrients, and at this time concentration of potassium dihydrogen phosphate would be effective. The increase of KH_2PO_4 from %0.4 to %0.8 would increase the pectin esterase activity in 24-48 hours of fermentation time, but it has the most effect in 48 hours .so, enzyme activity in 48 hours and %0.8 concentration of KH_2PO_4 would have the most value .ANOVA analysis showed the ammonium sulphate in range of %0.25 -%0.45,and PH in range of 4.2 to 6.18 have insignificant effect on enzyme activity .

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