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The effect of citrus pulp type on pectinase production in solid-state fermentation: Process evaluation and optimization by Taguchi design of experimental (DOE) methodology

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ABSTRACT

Pectinase is an important enzyme that finds application in many food processing industries and solid state fermentation (SSF) is an attractive technology for enzyme production. In this work, design of experimental (DOE) methodology using Taguchi orthogonal array (OA) was applied to evaluate the influence of five factors (different levels of citrus pulp, initial pH of the medium, C/N ratio, type of solid substrate and citrus pulp) on the pectinase production by *Aspergillus niger* under solid-state fermentation. The results showed that citrus pulp concentration, type of solid substrate and citrus pulp were found to be the most effective factor for promoting enzyme production and the supplementation of the medium with citrus pulp caused a 23% increase in the pectinase production when compared with the basal medium. The study shows that the Taguchi's method is suitable to optimize the experiments for the production of pectinase (R^2 = 0.946).

Key words: pectinase production, citrus pulp, design of experiment; Taguchi orthogonal array, solid-state fermentation

Introduction

Pectinase or pectinolytic enzymes are enzymes, which degrade pectin substances and are of great importance to the food industry. It has been reported that microbial pectinases account for 25% of the global food enzymes sales. These enzymes have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater (Jayani et al., 2005). The best-known microbial producers of pectinase are different species of *Aspergillus* fungi and, among the commercial pectinolytic enzymes, preparations obtained by the industrial cultivation of *Aspergillus niger* are some of the most popular (Debing et al., 2006).

An alternative for the production of these enzymes is solid-state fermentation (SSF). SSF plays an important role, and has a great perspective for the use and bioconversion of different agro-industrial residues such as citrus pulp. This material is rich in pectin that acts as the inducer and support, and thus, it can be used as substrate for the production of pectinolytic enzymes by microorganisms.

Utilization of agro-industrial residues for enzymes production using SSF minimizes the pollution and allows obtaining high added value products using an economical technology. Citrus pulp is the main solid by-product resulting from processing industry citrus.

As per FAO statistics (Graziano da Silva, 2013), the citrus production in Iran was about 4.6 million tons. It gives an estimation of about 920 000 tons of citrus pulp produced per year. Also, Guilan province is the largest rice producer in Iran. Rice cultivation area in Guilan is over 230 000 ha with an average farm yield of 6.3 tons/ha and an approximate straw production of 3.5 tons/ha. Potentially combination with agrochemical wastes is a cheap and valuable source as growth media for different biotechnological processes including microbial enzymes production (Khayati et al., 2013).

According to several authors (Martins et al., 2002; Silva et al., 2002; Silva et al., 2005) the production of pectinase on solid substrate, such as agricultural residues, is affected by culture conditions. Statistical experimental designs such as Taguchi orthogonal design can collectively optimize all the

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affecting parameters to eliminate the limitations of a single-factor optimization process. The traditional method of optimization involves varying one factor at a time, while keeping the others constant. This strategy requires a relatively large number of experiments and frequently fails to anticipate the optimal conditions. This essential shortcoming is due to the inability of the approach to consider the effects of possible interactions between factors. The deficiency can be overcome by applying more efficient, statistically based experimental design. In this respect, Taguchi orthogonal design is important tools to determine the optimal process conditions. The advantages of using the Taguchi method are that many more factors can be screened and optimized simultaneously and much quantitative information can be extracted by only a few experimental trials. Therefore, these methods have been extensively applied in parameter optimization and process control (Anvari & Khayati, 2009).

The aim of the current study was to investigate the production of pectinase by *Aspergillus niger* PTCC 5010, by means of solid-state fermentation of a mixture of different citrus pulp with rice or wheat straw, and pectinase production fermentation factors optimization was performed using fractional factorial design of orthogonal array of Taguchi methodology. The L-16 experimental array data revealed that different fermentation factors interact with microbial system at individual and in association with other factors at interactive levels and contributes for enhancement of microbial enzyme production.

Materials and Methods

Microorganism and inoculums

Aspergillus niger PTCC 5010 was obtained from the Iranian Research Organizationfor Science and Technology (IROST) and maintained as stock culture on potato dextrose agar medium (potato 20%, dextrose 2%, agar 2%). Conidia were suspended in 0.1% Tween 80 and a volume equivalent to 10^7 spores per gram of substrate was used as inoculum.

Fermentation conditions

Rice or wheat straw with percentages of different of citrus pulp types were used as solid substrates for solid-state fermentation (SSF). Ten grams of mixed substrates taken in 500 ml Erlenmeyer flasks was moistened with mineral medium [7.6g $NaNO_3$; 3.0g KH_2PO_4; 1.5g $MgSO_4$; 1.5g KCl; 2.4g KH_2PO_4; 6.6g $(NH_4)_2SO_4$ and 0.4g $CaCl_2$ per liter] to reach a final moisture content of 70% (w/v), then initial pH of the medium was adjusted according to Table 1. After sterilization, the flasks were inoculated and incubated at 32±1ºC. After incubation period of fermentation, extraction of the enzyme was carried out according to Khayati & Kiani (2012) and the supernatant was used for analytical assays.

Table 1. *Experimental L16 orthogonal array and results the pectinase activity*

Exp. No.		**Factor levels**		**Enzyme activity (U/g)**		
Levels of citrus pulp		**initial pH**	**C:N ratio**	**solid sub.**	**type of citrus pulp**	
1	0	5	5	wheat straw	orange	110.01
2	0	6	10	wheat straw	orange	120.54
3	0	7	20	rice straw	lemon	124.06
4	0	8	40	rice straw	lemon	102.98
5	10	5	10	rice straw	lemon	129.80
6	10	6	5	rice straw	lemon	128.85
7	10	6	40	wheat straw	orange	111.60
8	10	7	20	wheat straw	orange	119.91
9	30	5	20	wheat straw	lemon	121.18
10	30	6	40	wheat straw	lemon	125.65
11	30	7	5	rice straw	orange	133.64
12	30	8	10	rice straw	orange	163.33
13	50	5	40	rice straw	orange	155.99
14	50	6	20	rice straw	orange	172.59
15	50	7	10	wheat straw	lemon	129.80
16	50	8	5	wheat straw	lemon	123.74

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Experimental design and statistical analysis

The Taguchi method applies fractional factorial experimental designs, called orthogonal arrays, to reduce the number of experiments while obtaining statistically meaningful and worthwhile results. The selection of a suitable orthogonal array depends on the number of control parameters and their levels (Behnajady et al., 2012). An experimental L16 array from the Taguchi method was applied for the optimization of the enzyme production. The five factors selected were: different percentages of citrus pulp (0, 10, 30 and 50% w/w), initial pH of the medium (5, 6, 7 and 8), C:N ratio (5, 10, 20 and 40), solid substrate (wheat straw and rice straw) and type of pulp (orange and lemon). The array of the experimental factors and the levels are given in Table 1. For each experimental trial of the independent variables in the experimental design, the dependent parameter (pectinase production) was determined. The L16 orthogonal array was selected by the Taguchi method. The number of experiments required is drastically reduced to 16. This means that 16 experiments with different combinations of the parameters should be conducted to study the main effects and interactions, which in the classical combination method using full factorial experimentation would require $4^3 \times 2^2 = 256$ experiments to capture the effective parameters.

Analysis of variance (ANOVA) was generated, and the effect of terms were determined. The significances of all terms were judged statistically by computing the *p*-value <0.05. The analysis of data and optimization process were generated using Minitab statistical software version 15.

Enzyme assay

The crude enzymatic extract was assayed for pectinase activity. Pectinase activity was determined by measuring the reduction groups liberated from polygalacturonic acid substrate. The reaction mixture contained 245 µL of polygalacturonic acid (dissolved in 50 mM acetate buffer, pH=4.5) and 245 µL of the enzyme extract. The reaction mixture was incubated at 37°C. After 30 min of incubation, the reducing sugars released were quantified (Ruiz et al., 2012). The standard curve was established using galacturonic acid as reducing sugar. One unit of pectinase activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute under the assay conditions.

Results and Discussion

Effects of independent variables on responses

About enzyme fermentation techniques, solid-state fermentation generally is preferred because it allows production of highly concentrated crude enzymes with consequently low costs for extraction of pure enzymes (De Gregorio et al., 2002). Solid-state fermentation holds tremendous potential for the production of enzymes (Patil & Dayanand, 2006a). The enzymes of microbial origin were found to be more advantageous than others. Biosynthesis of pectinase by fungi and other microorganisms depends on some factors, including the components of the nutrient medium, cultivation temperature, initial pH of the medium and etc. (Kashyap et al., 2003; Kuhad et al., 2004; Patil & Dayanand, 2006b). In the present study production of pectinase using a mixture of different citrus pulp with rice or wheat straw was carried out and the production conditions were optimized. It is essential to optimize the fermentation medium for cost-effective production of pectinase (Mukesh Kumar et al., 2012). Taguchi experimental design is a good positive option for the optimization of biotechnological processes for production of microbial enzymes (Anvari & Khayati, 2009). In this case, the influence of 5 factors i.e., different percentages of citrus pulp (% w/w), initial pH of the medium, C:N ratio, solid substrate and type of pulp chosen for optimization of pectinase production by *A. niger* PTCC 5010 in Taguchi experimental design in 16 runs. The results of Taguchi experimental design show the efficiency of pectinase production ranging from 102-172 U/g corresponding to the combined effect of the five factors in their specific ranges (Table 1). The main effects of each parameter are presented in Figure 1, which serve as a measure to view individual variables' contributions on the production of pectinase. This was estimated based on the averages of measurements made at the level of each factor.

The pH of the culture is one of the most important environmental parameters affecting microbial cell growth and enzyme production. Each microorganism possesses a unique optimum pH and pH range for its growth and activity. Filamentous fungi are supposed to thrive over a broad range of pH under solid-state culture, because the solid substrate holds a better buffering capacity (Shankar & Mulimani, 2007). The effect of initial pH on enzyme production was studied by adjusting the pH of medium from 5 to 8. Although pH 5–8 favored pectinase production, the maximum pectinase production (136.9 U/g of substrate) was observed when the initial pH of the growth medium was 6.0 (Figure 1A). However, pectinase production declined sharply from pH 6 to 7.The results indicated that pectinase production was significantly affected by pH variations; therefore, it is

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concluded from the present experimental results that adjustment of initial growth medium pH is necessary for maximum pectinase yield by *A. niger* PTCC 5010. Maximum pectinase production has been reported in different pH ranges (i.e. 5-8) for different fungi (Hours et al., 1998; Martins et al., 2002; Silva et al., 2005). pH requirements vary from species to species and even in different strains of the same species isolated from different habitats (Wang et al., 2005).

The C/N ratio of the medium in SSF has a great impact on the physical properties of the substrate (Casas López et al., 2003). In the present study, the effect of different C/N ratios on extracellular pectinase production by *A. niger* was studied. The highest pectinase production (135.9 U/g) was achieved at C/N ratio of 10:1 and a very high or low C/N ratio resulted in relatively lower yield of enzyme production (Figure 1B). However, the pectinase activity at the C/N ratio of 10:1 was not statistically different from that of the C/N ratio of 20:1. This result showed that the C/N tolerable limit for the enzyme production was not too narrow. A decrease in enzyme yield was observed with further increases in C/N ratio. A previous study showed that pectinase production of *A. niger* NCIM 548 with an optimum C/N ratio of 5.9 gave a much lower yield of enzyme activity (22.87 U/ml) (Yugandhar et al., 2008) than our results (124.1 U/g) (Figure 1B). This suggests that, among other chemical and physical factors, the C/N ratio is another important one that needs to be optimized in the main component of medium.

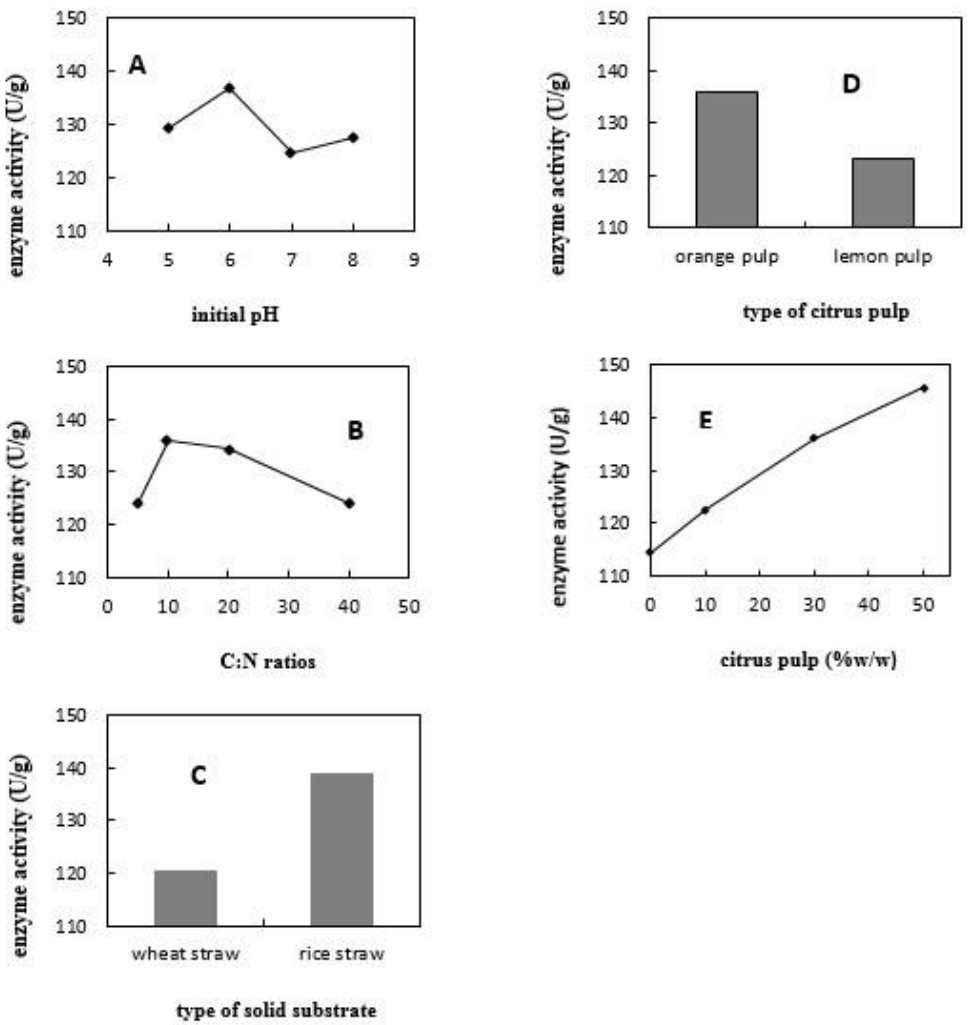

Figure 1. *Main effects of the variable for the production of pectinase based on the Taguchi experimental design result*

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The effect of different agro-industrial residues on pectinase production was studied by using a variety of solid substrates, since they act as support matrix and nutrient source for the production of enzymes. In this study, wheat straw or rice straw with citrus pulp were used as solid substrates for pectinase production by *A. niger*. These materials were chosen as solid stat substrate ingredients because they are low cost, extensively sourced, and nontoxic. Among these rice straw have been found to be the best and suitable for pectinase production and it has also been used for the production of lipase by *Rhizopus* sp. (Khayati et al., 2013; Khayati & Kiani, 2012). In this study, rice straw was a better substrate for pectinase production than wheat straw (15.5%) (Figure 1C). Many researchers reported that SSF processes are significantly influenced by the nature of solid substrate (Kashyap et al., 2003; Debing et al., 2006).

Several workers have studied the production of extracellular pectinases by *Aspergillus sp.* using pectic substrates or sugar beet pulp etc. The presence of pectic materials in the culture medium induced production (Aguilar & Huitron, 1990; Jain et al., 1990a; Jain et al., 1990b). Pectinase production from *A. niger* PTCC 5010 was carried out with different citrus pulp and the results are shown in Figure 1D. The results showed that enzyme production were in orange pulp was higher than lemon pulp. The chemical composition of the different citrus pulp is shown in Table 3. Orange pulp has high protein content, more particularly pectin (Table 3). This indicated that orange pulp might be a suitable inducer in compare with lemon pulp for pectinase production by solid state fermentation. Patil & Dayanand (2006b) also reported an improvement in pectinase yield by the adding of orange peel to the solid substrate as an inducer for the production of pectinolytic enzymes. However, medium supplementation could eventually improve enzyme production.

Selection of substrate depends upon several factors mainly related with cost and availability. Also, one of the most important parameters in fermentation systems is the level of substrate used. In this study, four different levels of citrus pulp varying from 0% (w/w) to 50% (w/w) were used. As shown in Figure 1E, with an increase in citrus pulp concentration pectinase production by *A. niger* was enhanced linearly, probably due to the nature of the induced effect is citrus pulp. Also, result of this study showed that the addition of citrus pulp into the culture medium, increased pectinase production by 23% compared with a medium without citrus pulp. Citrus pulp was also found to efficiently improve the production of extracellular pectinolytic enzyme by many other researchers (De Gregorio et al., 2002; Kuhad et al., 2004; Ruiz et al., 2012).

Analysis of the experimental design

The pectinase activity was found to range from 102 to 172 U/g in response to the variation in the experimental conditions (Table 1). The results from the analysis of the experimental design are shown in Table 2. The degree of significance of each factor is represented in Table 2 by its *p*-value; when a factor has a *p*-value of less than 0.05 it influences the process in a significant way for a confidence level of 0.95. The results obtained show that all of factors; different percentages of citrus pulp (% w/w), solid substrate and type of pulp except initial pH of the medium and C:N ratio were significant (p-value < 0.05). The results indicated that the citrus pulp levels in the culture media, type of solid substrate and citrus pulp were the major contributing factors to pectinase production.

Table 2. *Analysis of variance of the regression parameters for the Taguchi experimental design*

Source	D. F.	Sum of Square	Mean Square	*F*-value	*p*-value
Citrus pulp (% w/w)	3	2300.4	766.81	10.47	0.023
Initial pH	3	235.1	108.38	1.48	0.347
C/N ratio	3	496.7	165.55	2.26	0.224
Type of solid state	1	1383.9	1383.9	18.90	0.021
Type of citrus pulp	1	644.5	644.5	8.80	0.041
Residual Error	4	292.9	73.42		
Total	15	5443.5			

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Table 3. *Macronutrients composition of different citrus fruits on dry weight basis (Ali et al., 2010).*

Parameters (%)	Orange	Lemon	Grapefruit
Total ash	2.1	1.7	3.4
Crude fat	1.5	1.2	2.1
Crude fiber	8.6	5.7	8.2
Crude protein	4.2	2.2	2.4
Total sugar	16.5	12	15.1
Reducing sugar	12.4	10.2	11.2
Non-reducing sugar	4.2	2.5	4.1
Lignin	2.2	1.3	2.1
Pectin	12.8	4.4	8.1

The quality of the experimental of design developed was evaluated based on the value of coefficient of determination (R^2). Coefficient of determination (R^2) is defined as the ratio of the explained variation to the total variation and used to measure the degree of fitness. In this case, the R^2 value was 0.946, that Joglekar & May (1987) suggested for a good fit of a model, R^2 should be at least 0.80. This implied that 94.6% of the variations for the production of pectinase are explained by the independent variables and only 5.4% of the total variability in the response was not explained by the model. The relatively high value of R^2 (0.946) demonstrated a high degree of agreement between the experimental observations and predicted values (Figure 2) (Khayati & Kiani, 2012).

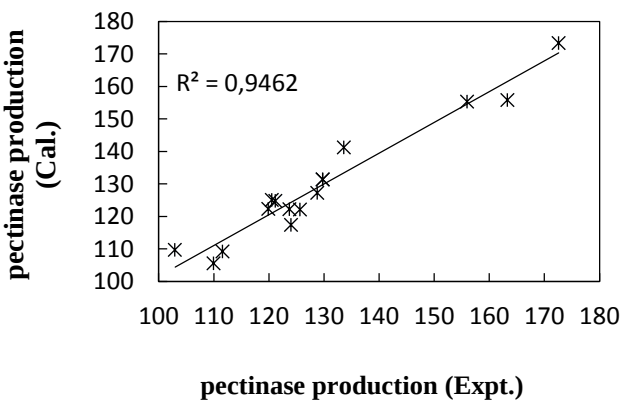

Figure 2. *The relationship between the calculated the production of pectinase and experimental data*

The residues were also examined for normal distribution. Fig. 3 shows the normal probability plot of residual values. It could be seen that the experimental points were reasonably aligned, thus suggesting normal distribution.

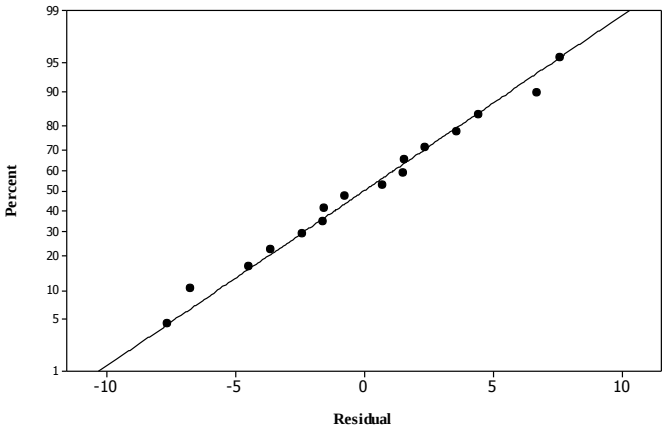

Figure 3. *Normal probability plot of residual values for the production of pectinase*

Conclusion

During the recent years, efforts have been directed to explore the means to reduce the enzyme production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for enzyme production (Khayati et al., 2013). Our finding showed that combination of orange pulp with rice straw could be used as suitable and low-cost substrate for pectinase production by *A. niger*. Also, the present investigation revealed that the Taguchi methodology provided a systematic and efficient mathematical approach to understand pectinase production for optimization of near optimum design parameters, only with a few well-defined experimental sets. Furthermore, we also demonstrated that citrus pulp 50% w/w, initial pH = 6, C/N ratio 10, rice straw as solid substrate and orane pulp the best conditions determined by the Taguchi method.

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